LONG-TERM MEMORY RULES WITHIN THE FEEDING NEURAL CIRCUIT OF THE MARINE SNAIL *APLYSIA CALIFORNICA*

A Thesis

by

ROBERT MUELLER

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This thesis meets the standards for scope and quality of
Texas A&M University-Corpus Christi and is hereby approved.

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December 2022

ABSTRACT

Long-term memory (LTM), which is memory that lasts for at least 24 h, is known to follow specific rules for formation and retention such as: 1) spaced training (S-T) protocols induce more persistent memory compared to massed training (M-T) protocols, 2) diurnal training (D-T) is more effective in inducing LTM than nocturnal (N-T) training, 3) LTM requires transcription of DNA into mRNA and translation of mRNA into new proteins. This project utilized the marine snail *Aplysia californica* to explore these LTM rules across two neural circuits that mediate both defensive and appetitive behaviors. Previous research in *Aplysia* revealed that repeated exposure to aversive stimuli induces an enhancement of defensive responses, known as long-term sensitization (LTS), as well as a decrease in feeding motivation, known as long-term feeding suppression (LTFS). These behavioral modifications are mediated at least in part by long-term increased excitability (LTIE) of sensory neurons, and long-term decreased excitability (LTDE) of decision-making neuron B51 (Shields-Johnson et al. 2013; Byrne and Hawkins, 2015). This project explored whether the feeding neural circuit in *Aplysia* follows the above LTM rules. Behavior results indicate that spaced training successfully induced both LTS and LTFS. However, massed training induced the expression of feeding suppression in the absence of sensitization, uncoupling these two normally co-expressed behavioral modifications. Results also revealed expected LTS and LTFS in the D-T group compared to D-UT group. However, nocturnal training did not induce either LTS or LTFS compared to the N-UT group. These findings indicate that nocturnal training is not conducive for LTM formation in both defensive and appetitive behaviors. Current electrophysiology experiments show a trend suggesting that B51 LTDE requires both transcription and translation for LTM formation. If the current trend continues, this result would indicate that learning-induced long-term plasticity within the feeding neural circuit of *Aplysia* requires

transcription and translation mechanisms analogous to those necessary for long-term plasticity within the defensive neural circuit. Collectively, these findings indicate the complex nature of LTM formation within the feeding neural circuit of *Aplysia* by revealing that feeding follows some of the above LTM rules, but not all of them. Specifically, massed training induced LTFS contradicts the LTM rule that massed training is not conducive for LTM formation. Therefore, this study filled a previous gap in knowledge in how feeding in *Aplysia* is mediated by a subset of general LTM rules. Future directions from this study will reveal further mechanisms for the formation and retention of sustained memories in *Aplysia* and the universality of memory across many different organisms.

DEDICATION

To the pursuit of knowledge…in the wise words of Dory: Just keep swimming!

ACKNOWLEDGEMENTS

As I wrap up this journey, I have so many incredible people to thank!

First and foremost, I want to thank my professor Dr. Riccardo Mozzachiodi! You have helped me learn and grow so much as a student, as a lab member, as a neurobiologist, and as a person. You have offered me so much guidance over these years for which I am forever grateful. You have such a vast wealth of knowledge and love for our work that you display daily. My favorite discussions have always been just you and I in your office talking about memory! You have facilitated my love and desire to dive even deeper into our memory research. So, thank you for taking a chance on me and working through all of my redundancies (I am the King of Redundancy).

Next, I want to thank Dr. Marcy Wainwright! From the very beginning, I have felt so incredibly welcomed into our lab and much of that is thanks to you. Your support and encouragement have never wavered even through some difficult times. We jokingly have said "Just keep swimming" for so long that it now has become such a part of my journey here. I cherish all of the conversations we have had, and you have taught me so much on how to be resilient and to keep pushing forward. Words will never be able to express my deepest gratitude toward you and Dr. Mozzachiodi! From the bottom of my heart, I thank you both! <3

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work and life could be. You always made me laugh whenever I needed it most, even when you didn't even realize. I cannot wait to see how far you go on your journey, Thanks for listening and learning from me. I so enjoyed getting to share all that I have learned with you in the hopes to make it that much easier for you. I learned from Xin that we are all in this together, so it was an honor to share all that I've learned with you. Best of luck man! To our undergraduates: Kristen Rebeles, Elizabeth Carrillo, and Aubrey Dauria. I truly could not have accomplished this project without you all. From your late-night training sessions, to helping me sort through my data, to allowing me to learn for you all as well. You all are so incredibly bright and have such wonderful futures ahead of all of you! So please keep in touch as I wish you all the very best. Team Snails! :)

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To my late guinea, Coach: My little buddy. You were my best companion for so many years. I used to talk to you about life, love, snails, and so much more. All while you just wanted pets and to share my food. But you are deeply missed, and I hope I have made you proud. You and I against the world Coach!

And to my partner Jordana, my journey here is not complete without you! Never in my wildest dreams would I have thought that I would be leaving TAMU-CC with so much. I thank you for listening to all of my snail talks, for being my rock, for supporting me throughout so many hardships, for continuing to laugh with me, and even for the days we cried together, I'm so grateful to have been on this journey with you. I am so proud of all that we have accomplished, and we have so much more ahead of us. The best is yet to come, and I cannot wait to see where life takes us!

Obtaining my master's degree in Marine Biology has been a dream of mine since I was a little kid. So, to be here now at the end of my journey, I am blessed and forever grateful for my time here at TAMU-CC. Getting to continue my research in memory while also studying marine biology was a dream! I am forever grateful for you all.

With all my love,

Robert

P.S. Just keep swimming…

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ABBREVIATIONS

5-HT: Serotonin

ACT-D: Actinomycin D

ANI: Anisomycin

ARM: Anesthesia-resistant memory

ASW: Artificial seawater

BMP: Buccal motor program

B.n.2,3: Buccal nerves 2,3

BT: Burst threshold

cAMP: Cyclic adenosine monophosphate

cGMP: Cyclic guanosine monophosphate

CREB: cAMP-response element binding protein

D-T: Diurnal-trained

D-UT: Diurnal-untrained

FMRFamide: Phe-Met-Arg-Phe-NH_2

LTDE: Long-term decreased excitability

LTFS: Long-term feeding suppression

LTH: Long-term-hyper-excitability

LTIE: Long-term increased excitability

LTM: Long-term memory

LTS: Long-term sensitization

M-T: Massed-trained

M-UT: Massed-untrained

MN: Motor neurons

N-T: Nocturnal-trained

N-UT: Nocturnal-untrained

NO: Nitric oxide

P8/P9: Pedal nerve 8/9

PKA: Protein kinase A

PKC: Protein kinase C

PKG: Protein kinase G

R_m: Input resistance

S-T: Spaced-trained

S-UT: Spaced-untrained

STM: Short-term memory

SWE: Seaweed extract

T-ACT.D: Trained-Actinomycin-D

T-ANI: Trained-Anisomycin

T-V: Trained-vehicle

UT-ACT.D: Untrained-Actinomycin-D

UT-ANI: Untrained-Anisomycin

UT-V: Untrained-vehicle

TSN: Tail sensory neuron

TSWR: Tail-induced siphon withdrawal reflex

V_m: Resting membrane potential

ZT: Zeitgeber time

CHAPTER I: INTRODUCTION

The underlying mechanisms of learning and memory have perplexed scientists for decades. How do we form memories? How is long-term memory (LTM) distinguished from short-term memory (STM)? What cellular mechanisms drive LTM? This project focused on **behavioral and cellular components of LTM formation and retention** in the marine snail *Aplysia californica*. Past research in numerous organisms has given rise to general LTM learning rules: 1) spaced training protocols (i.e., training with repeated stimuli spaced apart) induce more persistent memory compared to mass training protocols (i.e., training with repeated stimuli in a compressed time frame; Josselyn et al., 2001). 2) In diurnal organisms, training is more effective in inducing LTM when it is administered during the day (i.e., diurnal training) than when it is delivered at night (i.e., nocturnal training; Rawashdeh et al., 2018). 3) LTM requires transcription of DNA into mRNA and translation of mRNA into new proteins (Sudhakaran and Ramaswami, 2017). In *Aplysia*, training protocols consisting of repeated aversive stimuli delivered during the day induce an increase in defensive responses (known as long-term sensitization) coupled with a decrease in the non-defensive behavior of feeding (long-term feeding suppression) lasting at least 24 h (Fernandez et al., 2003, Acheampong et al., 2012). However, a recent study challenged these results by utilizing protocols that did not induce long-term sensitization (LTS) but did induce long-term feeding suppression (LTFS; Mac Leod et al., 2018). These protocols decoupled the change in defensive response and the change in feeding behavior, which suggests that feeding may not adhere to LTM rules. Previous research in *Aplysia* has also shown these behavioral modifications are sustained, at least in part, by changes in the excitability of neurons B51 and tail sensory neurons. LTS, which is sustained by an increase in tail sensory neuron excitability, is known to require both transcription of DNA into mRNA and translation of mRNA into new proteins for LTM formation

(Montarolo et al., 1986; Byrne and Hawkins, 2015). However, the gap in knowledge was to determine whether the feeding neural circuit requires similar mechanisms as the defensive neural circuit for LTM.

Thus, this project explored whether the feeding neural circuit in *Aplysia* follows the above LTM rules by elucidating whether LTFS is induced by massed and/or nocturnal training (**Objective 1**) and whether its cellular mechanisms require transcription and/or translation (**Objective 2**).

Background and relevance

Reductionistic approach to researching memory – Aplysia californica

The human brain has between 19 billion and 23 billion neurons (Pakkenberg and Gundersen, 1997), which can create numerous difficulties when studying individual neurons or understanding the mechanisms driving learning and memory. A reductionist approach can be used to address difficult questions regarding memory formation and retention by researching elementary forms of learning which have evolved across many organisms (Carew, 2000; Kandel, 2001). Ubiquitous behavioral and cellular mechanisms, such as LTM rules, can be investigated more effectively with a reductionist approach because it allows for the study of the simple instances of memory storage in tractable animal models at the behavioral and cellular levels (Kandel, 2001). Extensive research has investigated the neurobiology of memory in *Aplysia* because this model system allows for the research of complex behavioral and cellular mechanisms in a simplified neural system (e.g., Kandel, 2001; Mac Leod et al., 2018, Farruggella et al., 2019).

Measurable behaviors

Aplysia exhibit measurable non-defensive behaviors such as **feeding** (Figure 1), and defensive responses such as the **tail-induced siphon withdrawal reflex** (**TSWR**, Figure 2; Kandel, 2001, Carew, 2000, Acheampong et al., 2012). During feeding, bites consist of full cycles of radula protraction, closure, and retraction, which leads to the consumption of food (Figure 1; Mozzachiodi et al., 2013). Following a mild stimulus to the tail, the animal exhibits an increase in

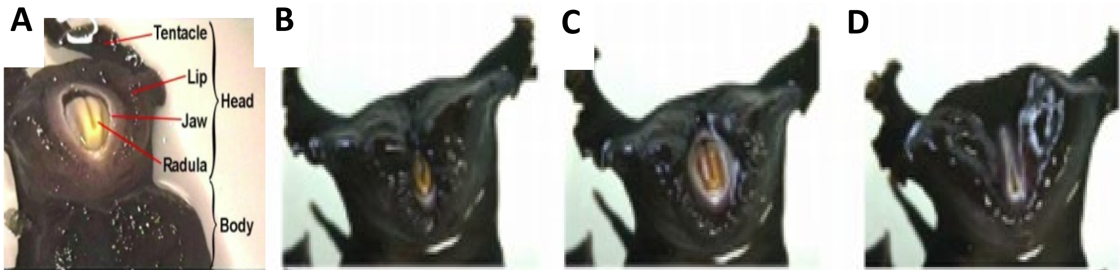

Figure 1: Illustration of feeding behavior in *Aplysia*. (A) The anatomy of the *Aplysia* mouth, which includes the lips, jaw, and radula. (B) In the presence of food, *Aplysia* will open its lips to expose the radula. (C) Protraction of radula. (D) Closure and retraction of the radula (From Brembs et al., 2002 and Nargeot and Simmers, 2012).

defensive behaviors by contracting the tail and the siphon into the body cavity (TSWR), which can be measured by timing the duration of the contraction (Figure 2; Cleary et al., 1998).

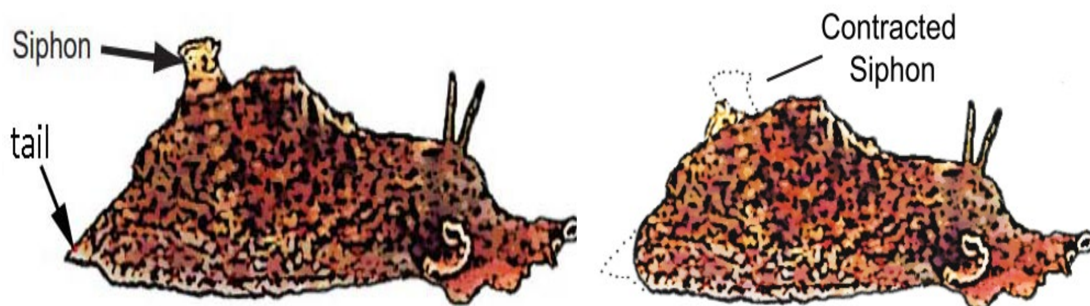

Figure 2: Illustration of the defensive behavior in *Aplysia*. In response to a mild stimulus to the tail (left), the animal contracts its tail and siphon (right). The duration of TSWR was measured by timing the onset of the siphon contraction to the first sign of relaxation back to a normal state. (Courtesy of Dr. Mozzachiodi)

Learned behavioral modifications

Following exposures to aversive stimuli (electrical shocks delivered to the body wall of the animal), *Aplysia* exhibit learned behavioral changes including feeding suppression and sensitization (Acheampong et al., 2012). **Feeding suppression (FS)** manifests as a decreased number of bites (Acheampong et al., 2012). **Sensitization,** which is an elementary form of learned fear, manifests as an increase of TSWR duration (Kandel, 2001).

The amount and pattern of aversive exposure affect the duration of learned behavioral modifications. Following aversive training consisting of repeated trials, the animal exhibited concurrent **long-term feeding suppression (LTFS) and long-term sensitization** (**LTS**; Acheampong et al., 2012). After four trials of aversive stimuli, delivered over a period of 1.5-h, LTFS and LTS were both observed 24 h after training (Figure 3; Acheampong et al., 2012, Mac Leod et al., 2018).

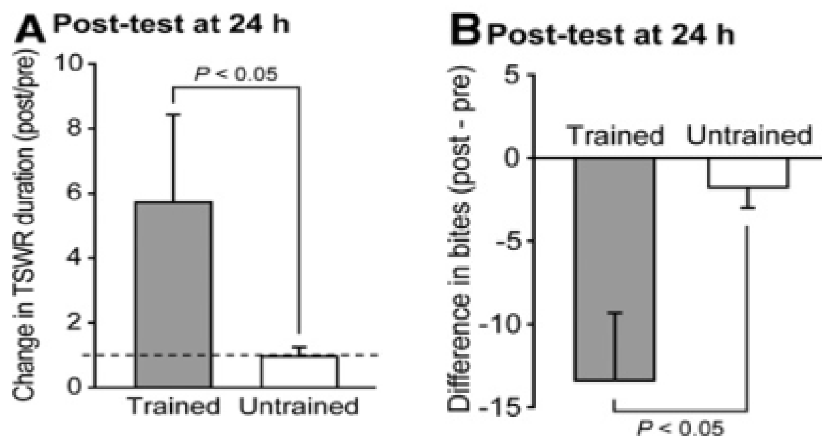

Figure 3: Four-trial training produced long-term sensitization (LTS) and long-term feeding suppression (LTFS) at 24 h post-test. (From Acheampong et al., 2012)

Decoupling feeding suppression and sensitization

Repetition and spacing of the stimuli may play a vital role in the duration of memory for LTS, but not necessarily for LTFS. Following a 4-day training with spaced stimuli (Figure 4A; delivered over a period of 1.5-h for 4 days), LTFS did not persist past 24 h but LTS did, indicating that feeding resumed earlier than defensive behaviors (Figure 4B; Mac Leod et al., 2018). Thus, LTFS exhibits a different time course than LTS, suggesting that LTFS may not follow LTM rules.

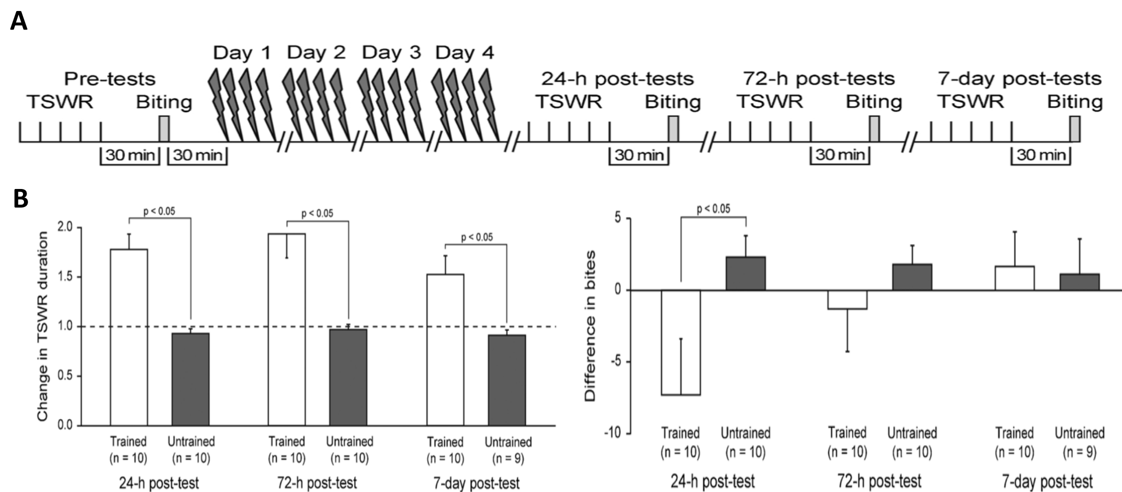

Figure 4: (A) Illustration of the spaced aversive training used in our lab. (B) Training induced LTS that lasted up to 7 days (left), but LTFS that was only present 24 hours after training (right; From Mac Leod et al., 2018).

Another experiment also indicated that LTFS can be decoupled from LTS in *Aplysia*. Untrained and trained animals were deprived of food for 2 or 14 days to investigate the effects of prolonged food deprivation on learning and memory (Figure: 5A, 5B; Mac Leod et al., 2018). Although food deprivation for 14 days prevented LTS in trained animals, LTFS was still observed in an attenuated form at the 24 h post-test, suggesting that feeding suppression can occur without sensitization (Figure 5C; Mac Leod et al., 2018). Food deprivation blocked memory formation for sensitization, which is consistent with previous work in the fruit fly *Drosophila* (Plaçais and Preat,

2013). Under food shortage, the brain self-allocates available resources and trims selected costly processes such as memory formation (Plaçais and Preat, 2013). Thus, these results further suggest that feeding suppression may not be following LTM rules.

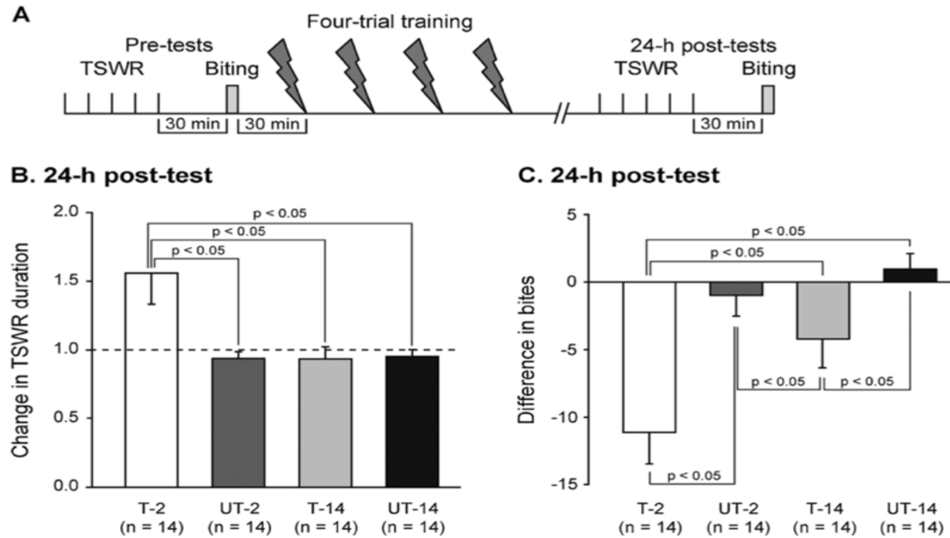

Figure 5: In 14-day food deprived (T-14) animals, four-trial training (A) does not induce LTS (B) but produces LTFS (C). (From Mac Leod et al., 2018)

Spaced vs. massed training

One known LTM rule is that spaced training is more effective for LTM formation and retention than massed training (Carew et al., 2001, Scharf et al., 2002, Wainwright et al., 2002). The main difference between spaced vs, massed training is temporal spacing of training. For instance, spaced training consists of multiple days of training whereas massed training consists of the same total amount of training, but instead of being spaced over multiple days, massed training is compressed over one day of training. Previous research indicated that four-day spaced training induced LTS in *Aplysia*, however, massed training protocols failed to induce LTS entirely in *Aplysia* (Wainwright et al., 2002). Repeated spaced trials appear to be more effective than massed trials for learning in the defensive TSWR. Massed training has been shown not to induce LTS, but the effects of massed training on LTFS had not been explored.

Diurnal vs. nocturnal training

Another general LTM rule is that diurnal training is more effective for LTM formation and retention than nocturnal training in *Aplysia*, which is a diurnal animal (Fernandez et al., 2003, Lyons et al., 2006). The circadian clock appears to regulate LTM formation in *Aplysia* based on previous work which revealed that training conducted during the day (11 am and 5 pm) induced LTS in *Aplysia,* whereas nocturnal training (11 pm and 5 am) did not induce LTS (Lyons et al., 2006). Although nocturnal training does not induce LTS, it had not been explored whether nocturnal training also inhibits LTFS.

Physiology of TSWR and feeding neural circuits

The extensive previous research on *Aplysia* neural circuits allows for the study of memory at the cellular and molecular levels (Mozzachiodi et al., 2013, Byrne and Hawkins 2015). An in vitro reduced preparation developed in Dr. Mozzachiodi's lab, which consists of buccal, cerebral, and pleural-pedal ganglia contains the neural circuits controlling feeding and the TSWR (Weisz et al., 2017).

Neuron B51

Neurons B51 (Figure 6) are a pair of cells in the buccal ganglia (one in each ganglion), which exhibit decision-making features for feeding behavior by switching from an inactive state to an active state during the occurrence of ingestive buccal motor programs (Figure 7; Nargeot and Simmers, 2012, Mozzachiodi et al., 2013, Dickinson et al., 2015, Weisz et al., 2017). B51 expresses an all-or-nothing burst of action potentials in response to stimuli known as plateau potential (Plummer and Kirk, 1990, Nargeot and Simmers, 2012). Activation of B51 instructs the feeding neural circuit to generate the neuronal activity that generates bites. Therefore, B51 activity can be used as an *in vitro* readout of feeding (Nargeot et al., 1999a).

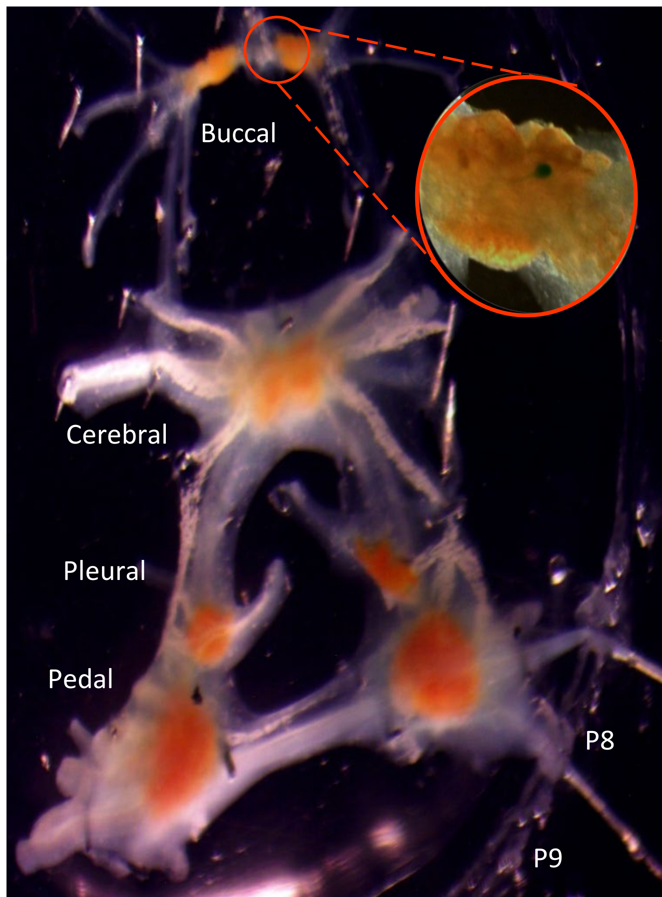

Figure 6: Picture of the reduced preparation utilized. Zoomed in on the buccal ganglia, which contains the feeding neural circuit and neuron B51 (highlighted green cell).

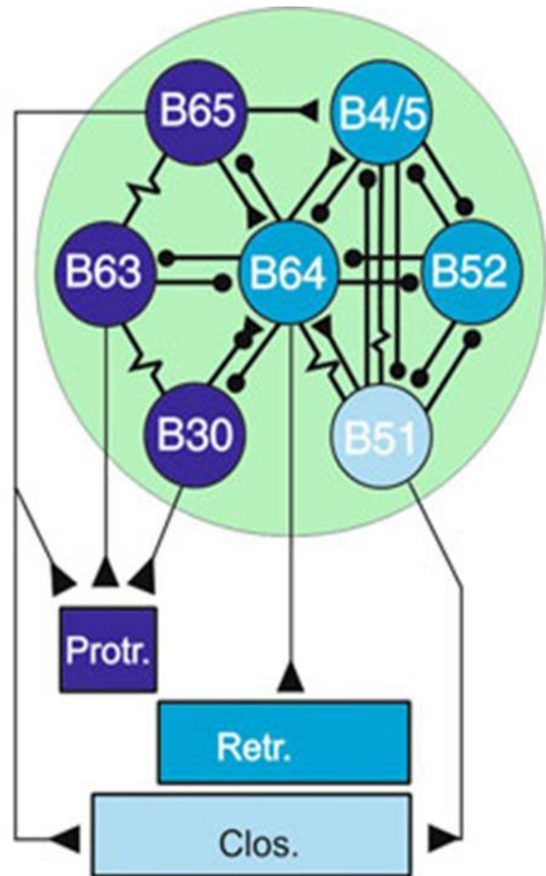

Figure 7: Neural circuits containing feeding central pattern generator (CPG). The CPG includes neurons responsible for radula protraction (dark blue), closure (light blue), and retraction (blue) including neuron B51. (Nargeot and Simmers, 2012).

Tail sensory neurons

Tail sensory neurons (TSNs; Figure 8) in the pleural ganglia activate motor neurons (MN) in the pedal ganglia, which contract the tail and indirectly mediate the siphon withdrawal, producing the TSWR (Figure 9; Cleary et al., 1998). Therefore, TSN activity can be used as an *in vitro* readout of the TSWR behavior (Cleary et al., 1998).

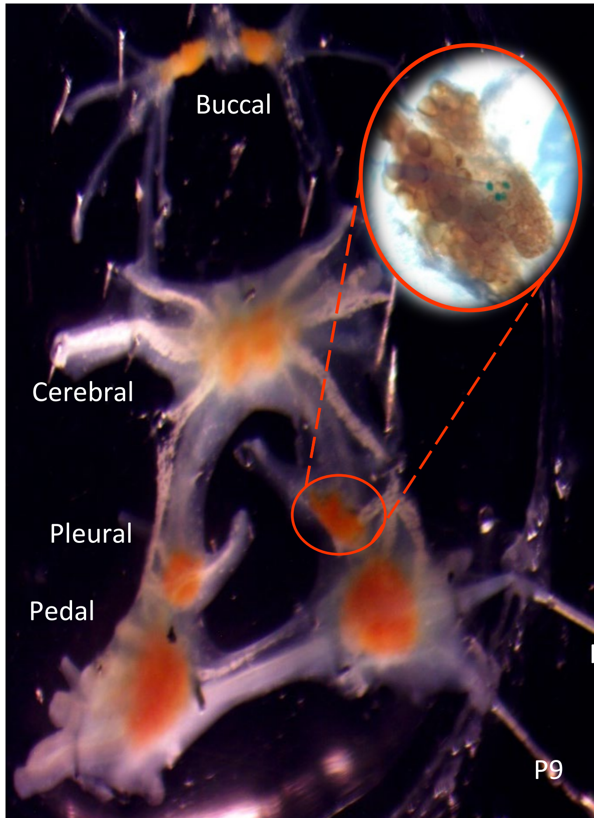

Figure 8: Picture of the reduced preparation utilized. Zoomed in on the pleural ganglia, which contains the defensive neural circuit and the TSNs that mediate the TSWR (highlighted green cells).

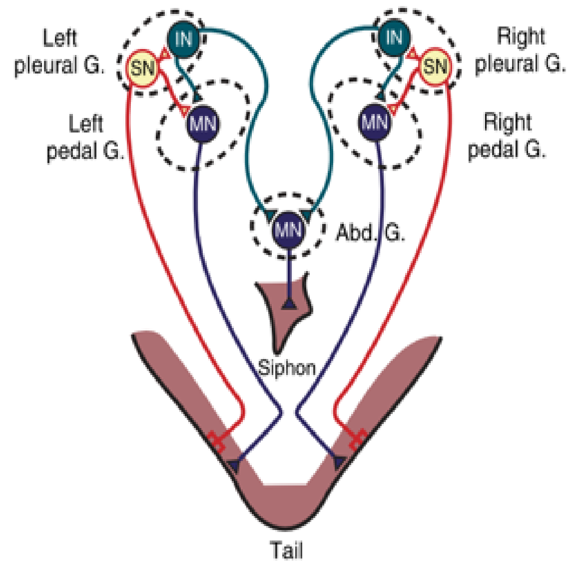

Figure 9: Neural circuits responsible for TSWR. TSWR is mediated, at least in part, by TSNs (SN; sensory neuron) in the pleural ganglion (G). TSNs activate a polysynaptic pathway via interneurons (IN) that project to motor neurons (MN) generating contraction of the tail and siphon. (From Byrne et al., 2008)

Neuronal excitability

Excitability is a neurophysiological parameter that assesses the sensitivity of neurons to inputs (Mozzachiodi and Byrne, 2010). Excitability in B51 is assessed by measuring its burst threshold, which is defined as the minimum amount of depolarizing current necessary to elicit a plateau potential (Figure 10; Nargeot et al., 1999a, Weisz et al., 2017, Chatterji et al., 2020). The excitability of TSNs is measured by the number of action potentials generated by a 1s, 2 nA injected current (Figure 11; Weisz et al., 2017).

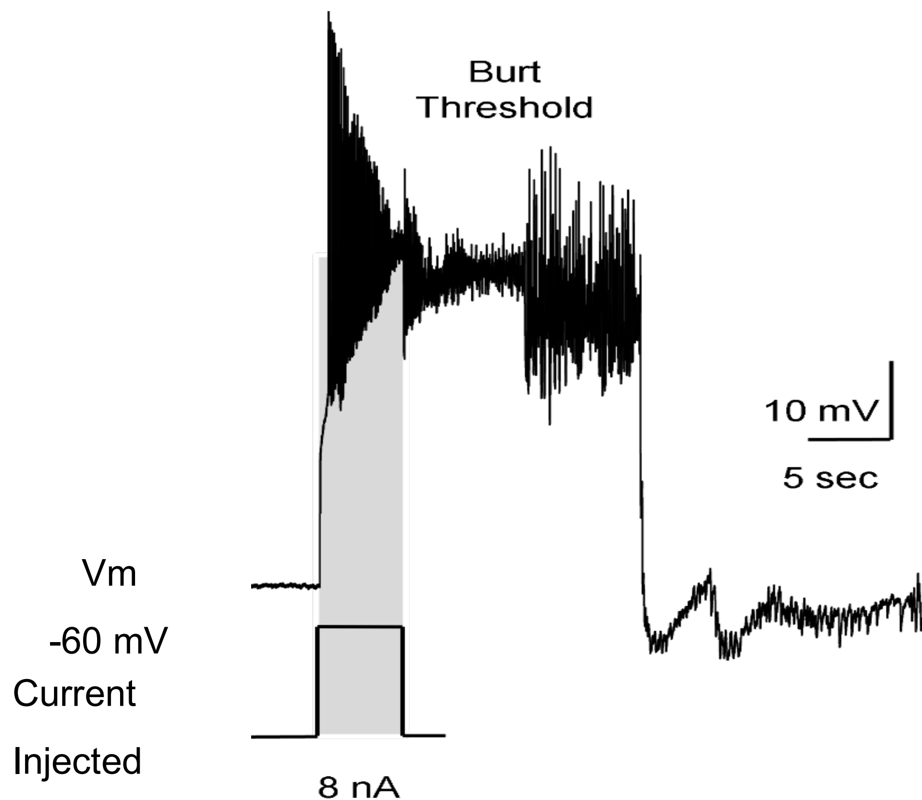

Figure 10: Excitability of B51 was measured by determining the burst threshold (BT) that outlasted the injected current (grey rectangle indicating 5 sec, 8 nA, depolarizing current). In the example illustrated, BT was 8 nA.

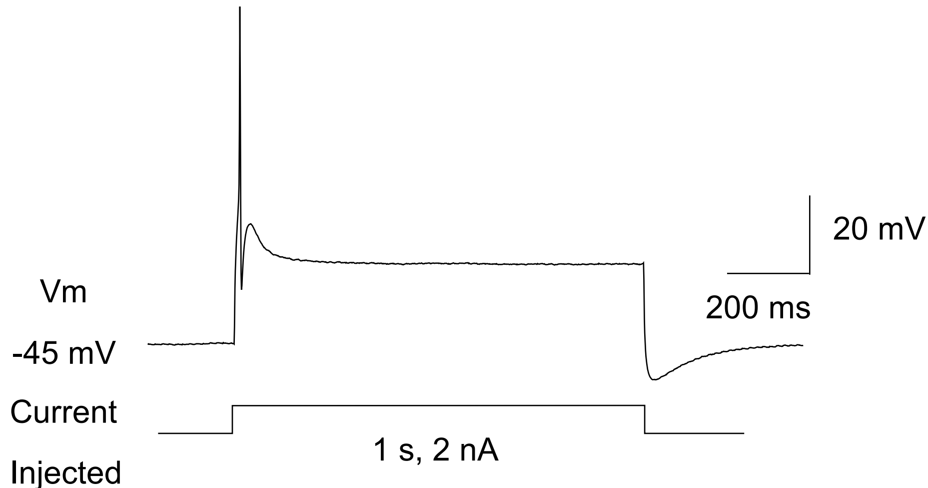

Figure 11: Tail sensory neuron excitability was measured by injecting a 1-sec, 2 nA pulse of depolarizing current and counting the number of action potentials evoked (1 action potential in example above).

Learning-induced cellular changes

Previous studies in *Aplysia* indicate that LTFS and LTS are sustained, at least in part, by changes in the excitability of B51 and TSNs. In particular, B51 **long-term decreased excitability (B51 LTDE)** and **TSN long-term increased excitability (TSN LTIE)** are correlates of LTFS and LTS, respectively (Cleary et al., 1998, Shields-Johnson et al., 2013). These cellular changes can

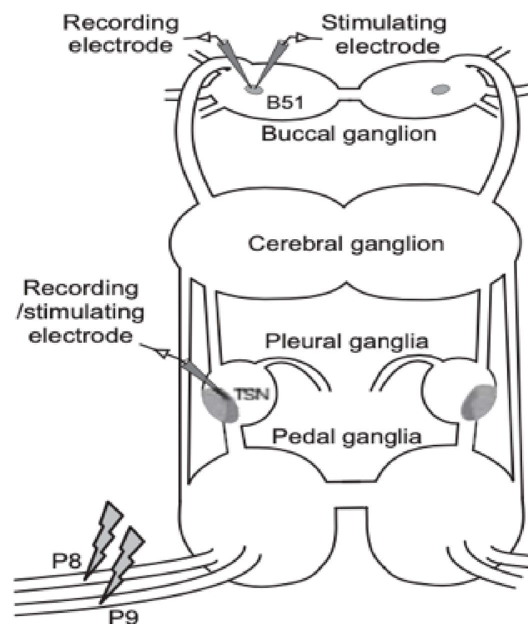

Figure 12: Configuration of *in vitro* preparation (Weisz et al., 2017).

be induced in the reduced preparation described in Figure 12 by using repeated electrical stimulation of the afferent pedal nerves P8 and P9, which mimics *in vitro* the aversive training utilized *in vivo* (Zhang et al., 1994, Weisz et al., 2017). B51 LTDE manifests as an increased burst threshold (Weisz et al., 2017; Figure 13A). Decreased excitability in B51 would lead to a reduced number of bites, as observed in LTFS *in vivo.* TSN LTIE manifests as an increased number of spikes generated from the injected current (Weisz et al., 2017; Figure 13B). An increased firing of TSNs would lead to an enhanced TSWR (Walters and Byrne, 1983), as observed in LTS *in vivo*.

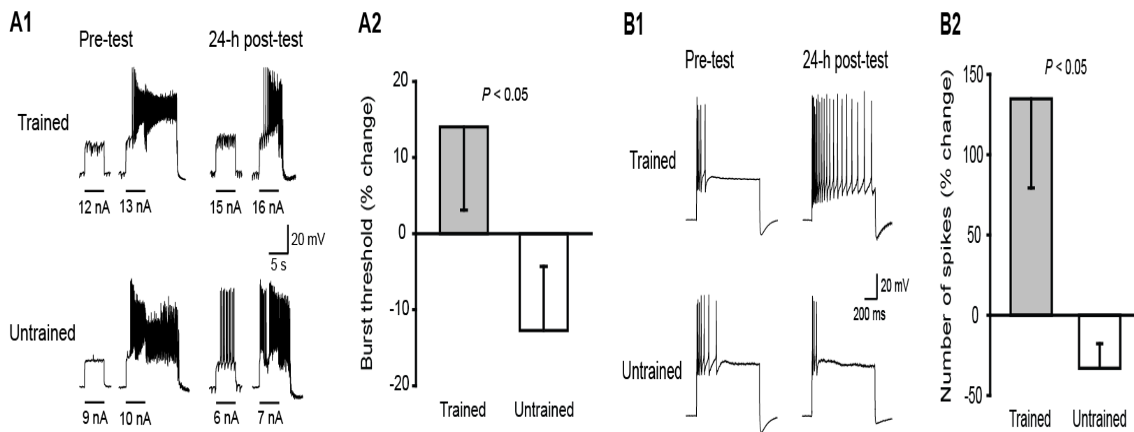

Figure 13: Four trial *in vitro* training induced decreased B51 excitability (increased burst threshold) and increased TSN excitability. A1) Decreased B51 excitability indicated by an increase to burst threshold. A2) Summary data of decreased B51 excitability. B1) Increased number of action potential spikes in TSNs. B2) Summary data of increased TSN excitability. (From Weisz et al., 2017)

Molecular requirements for LTM formation

Previous studies on *Aplysia* revealed that LTS and its cellular correlates, including TSN LTIE, depend on both transcription of DNA into mRNA and translation of mRNA into new proteins (Figure 14; Montarolo et al., 1986, Byrne and Hawkins, 2015). Transcription and translation are known to be ubiquitous molecular requirements for LTM formation (Tully et al., 1994, Quevedo et al., 2004). Transcription is the process of making an mRNA copy of a gene sequence that can ultimately be translated to encode new proteins (Montarolo et al., 1986, Clancy

and Brown, 2008, Byrne and Hawkins, 2015). However, it is currently unknown whether B51 LTDE also requires both transcription and translation.

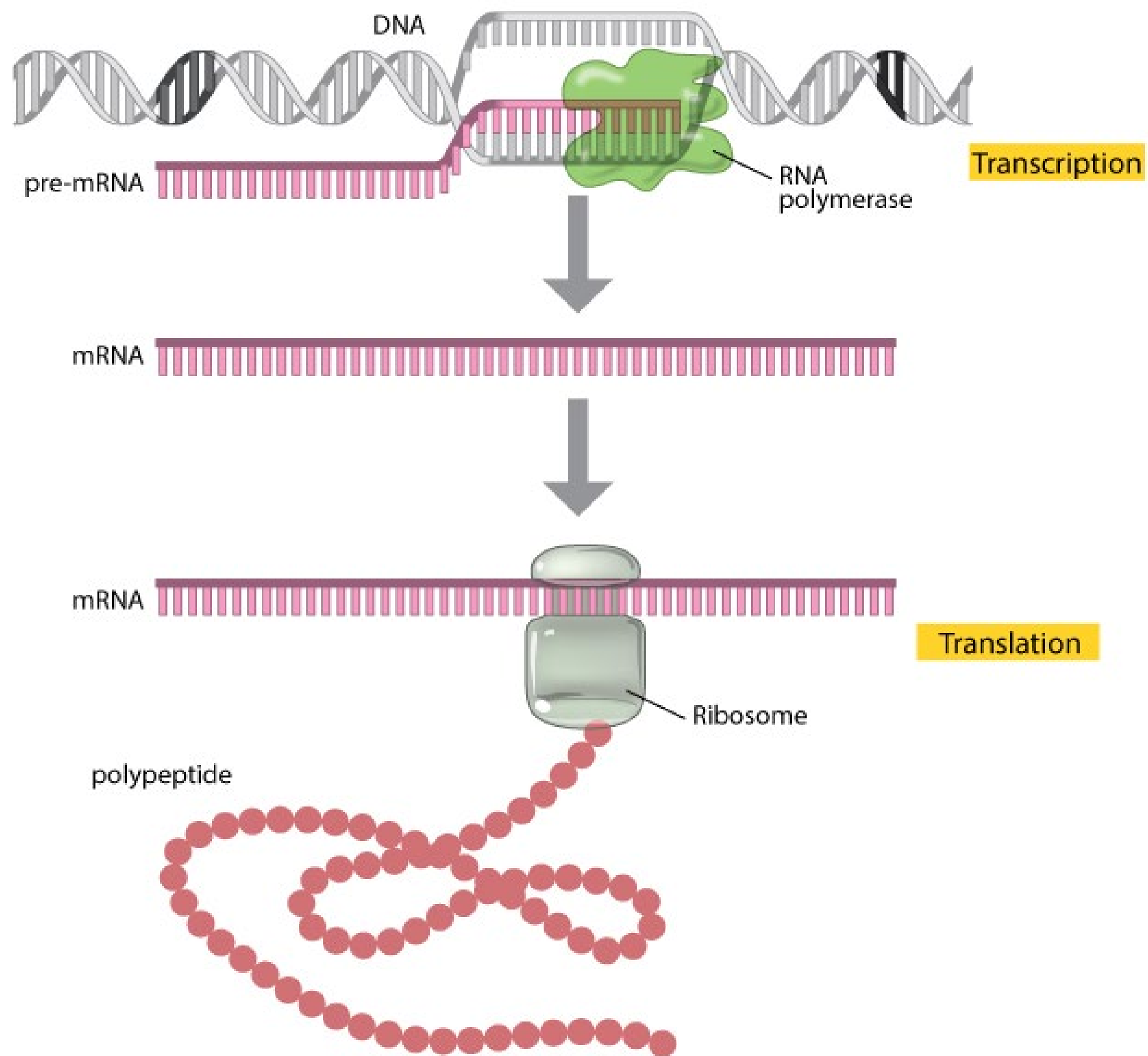

Figure 14: Illustration of transcription and translation processes. Transcription involves the enzyme RNA polymerase (green) which transcribes the DNA to produce an mRNA transcript (pink). The mRNA can then be translated to encode new proteins (Clancy and Brown, 2008).

CHAPTER II: OBJECTIVES

This project focused on whether the training-induced changes in feeding and its underlying neural circuit follow LTM learning rules analogous to those observed in the TSWR circuit.

Objective 1: Effect of training protocols that do not induce LTS on LTFS

The goal of this objective was to investigate whether LTFS is expressed following two protocols that are known not to induce LTS. Specifically, I explored the effects of massed training (Wainwright et al., 2002; **Objective 1a**) and nocturnal training (Fernandez et al., 2003; **Objective 1b**) on LTFS in *Aplysia*.

Objective 1a: Effect of massed training on LTFS induction

The goal of this objective was to explore the effects of massed training on LTFS. Massed training is known not to induce LTS, however, this objective determined whether massed training could induce LTFS in the absence of sensitization.

Objective 1b: Effect of nocturnal training on LTFS induction

The goal of this objective was to determine the effects of nocturnal training on LTFS. Specifically, this objective explored if the circadian rhythm modulates feeding suppression.

Objective 2: Role of transcription and translation in B51 LTDE

This objective investigated whether B51 LTDE is transcription and/or translation dependent. I utilized known blockers of transcription (Actinomycin D: ACT-D) and translation (Anisomycin: ANI) to examine if B51 LTDE requires protein synthesis for LTM formation and retention similar to TSN LTIE. Training-induced TSN LTIE served as a positive control for the effectiveness of the transcription and translation blockers (Montarolo et al., 1986)

CHAPTER III: METHODS

General methods

Location and logistics

All experiments were conducted in Dr. Mozzachiodi's research laboratory at Texas A&M University – Corpus Christi. Animals were kept in tanks adjacent to our on-campus lab in Tidal Hall. According to the NIH Office of Laboratory Animal Welfare, because *Aplysia* are an invertebrate species, experiments do not fall under IACUC regulations. Adult *Aplysia* were obtained from South Coast Bio-Marine (San Pedro, CA) and from Marinus Scientific (Lakewood, CA) and were housed in two aquaria (Aquatic Enterprises INC., WA) of continuously circulating 15 ˚C aquarium seawater (Instant Ocean) Housed *Aplysia* are normally entrained to 12:12 h cycles of light and dark, which is represented using zeitgeber time (ZT; Fernandez et al., 2003). ZT starts at ZT 0, which corresponds to the start of lights-on in the lab at 8 am. Newly arriving animals were allowed to acclimate to the tank for at least a 3-day minimum before undergoing any experimental procedures. Each animal was fed 0.5 g of dried seaweed (Emerald Cove® Organic Pacific Nori; Great Eastern Sun, Asheville, NC) in its holding tank three times a week (Monday, Wednesday, and Saturday) to maintain a constant body weight. Prior to any experiments, animals were food deprived for 2 days to maintain consistent feeding motivation. Furthermore, if an animal laid eggs, and/or secreted ink and/or opaline prior to their behavioral testing, the animal was not used for a minimum of seven days to ensure the animal did not have prior sensitization or impaired health.

Figure 15: Animals housed in their separate holding containers located in Tidal Hall at Texas A&M University – Corpus Christi (Photo credit: Edgar De La Garza).

Methods – Objective 1

Animal preparations for behavioral experiments

All animals underwent a surgical procedure to remove the parapodium (i.e., parapodectomy; Figure 16). This procedure granted better visualization of the siphon and that allowed for proper measurement of the TSWR (Cleary et al., 1998, Farruggella et al., 2019). Animals were temporarily anesthetized by burying them under ice for 20 min (Acheampong et al., 2012; Farruggella et al., 2019). After 20 minutes under ice, the animals were checked to ensure they were properly anesthetized by touching the sensitive rhinophores and/or siphon. If the animal did not respond to either touch stimulus, the animal was considered fully anesthetized. The animal

was then placed on top of the ice to expose the parapodia. Once exposed, hemostats were used to clamp the base of the parapodium to prevent loss of hemolymph and then cut. The incision began at the posterior position of the parapodium and was then cut for approximately 3 cm in a straight line across the top portion of the parapodia to a point just above the siphon (Figure 16; Mac Leod et al., 2018). Once the excess parapodium was trimmed, the hemostat remained clasped onto the parapodium for about 30 s to allow the tissue to heal. The procedure was then repeated on the other side of the animal. After both parapodia were cut, the animal was then gently placed back into a bowl of artificial seawater (ASW) and allowed to fully recover from the anesthesia before being returned to the housing tank. The animal was considered fully recovered and awake once the animal began to move around and attached to the bowl. If the animal secreted ink or opaline at any time during the procedure or upon waking up, the animal was disqualified and was not used in the experiment. Each animal underwent a recovery period of 7 days after the parapodectomy before beginning any experimental procedures.

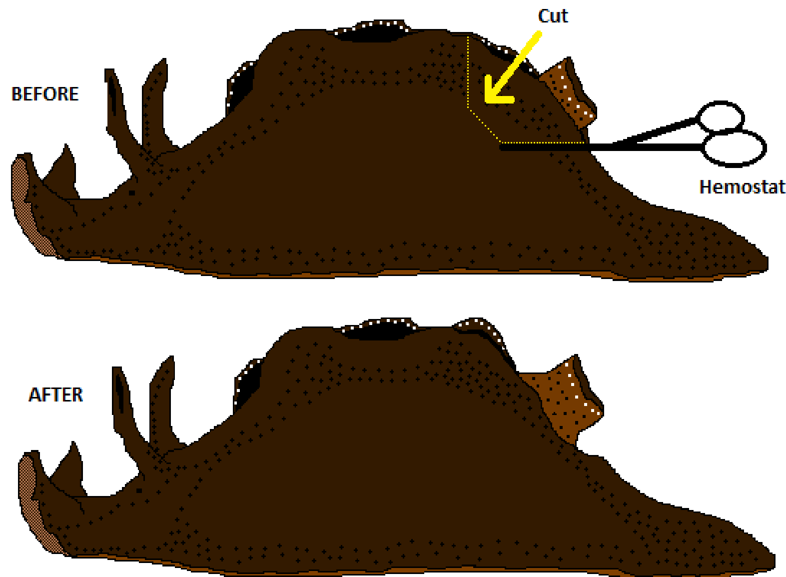

Figure 16: Illustration of parapodia before and after parapodectomy to grant better visualization of the siphon, which allowed for proper measurement of the TSWR (Courtesy of Dr. Mozzachiodi)

Behavioral measurements

TSWR - *During* TSWR testing, a hand-held bipolar electrode was used to deliver a mild electrical stimulus to the animal's side, inducing the TSWR (Mac Leod et al., 2018). A 300-400 ms, 2-mA electrical stimulus was applied to an identified a spot on the tail (Figure 17; Mac Leod et al., 2018). The duration of the TSWR was timed from the onset of the siphon contraction response to the onset of its relaxation. The average of five measurements was used to determine the TSWR in each animal (Mac Leod et al., 2018). TSWR duration was measured before (pre-test) training and then measured again (post-test) either at 24 h for **Objective 1a** or at 18 h for **Objective 1b** (see below). TSWR pre-tests for **Objective 1b** were conducted at different time points in the diurnal (ZT 4) and nocturnal groups (ZT 11 see details below).

Figure 17: A hand-held bipolar electrode was used to deliver a 2-mA electrical stimulus to an identified spot on the tail, inducing the TSWR.

Feeding - The same animals used to measure the TSWR were also used for the feeding response trials. A seaweed extract (SWE) was used to reliably elicit feeding behavior (i.e., bites) without the possibility of ingestion influencing the animal's response to behavioral stimuli (Acheampong et al., 2012; Farruggella et al., 2019). The SWE was prepared the day of the experiment to ensure freshness of the food stimulus. A half sheet of Emerald Cove® Organic Pacific Nori dried seaweed (about 10.5cm x 19.3cm) was cut into small pieces and then immersed in 300mL of ASW. The SWE solution was mixed for 30 min and then filtered through a coffee filter to collect excess pieces of seaweed. The final SWE was then stored in a 15°C incubator until it was needed for experimental use (Acheampong et al., 2012; Chatterji et al., 2020).

During pre-tests and post-tests, feeding was measured 30 min after TSWR measurements. The feeding response was measured by placing individual animals in a glass bowl containing 167 mL of SWE and 1333 mL of ASW for 1500 mL of SWE solution with a concentration of 1 part SWE to 8 parts ASW (Figure 18; Acheampong et al., 2012; Farruggella et al., 2019). Feeding was measured by counting the number of bites during a 5 min-period and video recorded. The 5 min duration for the feeding test began when the animal fully attached to the glass bowl. For a bite to be counted, it must have exhibited the full pattern of radula moments involved in ingestive biting behavior including radula protraction, closure, and retraction (Figure 1B-D; Farruggella et al., 2019). Feeding response was measured pre-test to establish a baseline for feeding behavior (pre-test). Feeding pre-tests for **Objective 1a** were conducted at the same time points for all groups, but **Objective 1b** pre-tests were conducted at different time points for diurnal (ZT 4) and nocturnal groups (ZT 11). Feeding response was then measured again (post-test) either at 24 h for **Objective 1a** or at 18 h for **Objective 1b** (see below).

Figure 18: An *Aplysia* exhibiting a bite while exposed to SWE during behavioral feeding test. The glass bowl ensures proper observation of *Aplysia* feeding behavior.

Behavioral training procedures

In *both* **Objective 1a** and **Objective 1b**, testing and training/treatments were conducted by different individuals, with the experimenter conducting the tests kept blind to the experimental history of the animals (e.g., Acheampong et al., 2012). Thus, training procedures were the same for all groups other than time of training. Following behavioral pre-test measurements, the animal was allowed to rest for 30 minutes before the onset of training. The trainer randomly determined whether the animal would be trained or untrained. Aversive training consisted of multiple trials of noxious electrical stimuli. Each comprised of 10 s train of ten 500-ms, 60-mA AC pulses, delivered using a hand-held probe to the animal's body wall (Figure 19; Acheampong et al., 2012). Training had to have caused the animal to release ink or opaline for training to have been considered successful (Figure 20). If the animal did not release ink or opaline in response to aversive training, the animal was excluded from the experiment.

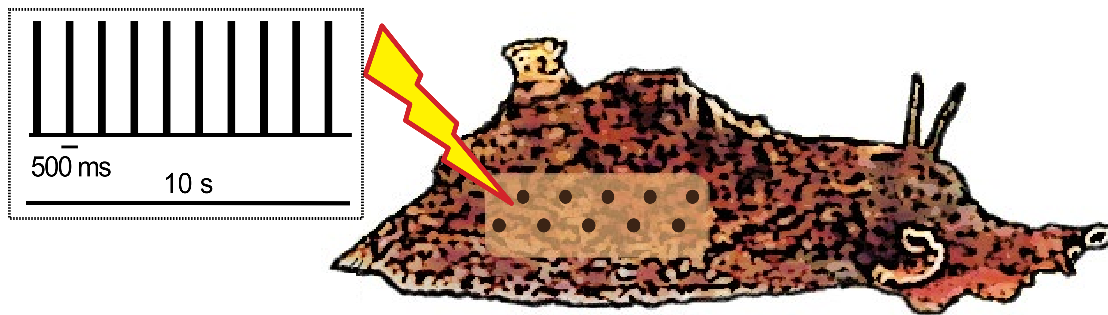

Figure 19: Illustration of a trial of aversive training applied to the body wall of the animal (Modified from Dr. Mozzachiodi)

Figure 20: Animal releasing ink indicating successful aversive training.

Methods – Objective 1a: Protocols for massed and spaced behavioral training

After the completion of TSWR and feeding pre-tests, animals were randomly subjected to one of the following training protocol groups: massed-trained (M-T), massed-untrained (M-UT), spaced-trained (S-T), or spaced-untrained (S-UT). Each group contained a 10-animal sample size. Massed training consisted of 16 trials in one single day administered every 30 min for a total of 7.5 h worth of training (Figure 21A; Wainwright et al., 2002). Spaced training consisted of 16 trials, 4 trials delivered each day with a 30 min inter-trial interval, distributed over 4 consecutive days (Figure 21B; Wainwright et al., 2002, Mac Leod et al., 2018). Spaced training, which is known to induce LTS and LTFS, was utilized as a positive control for training-induced behavioral changes. The untrained control groups underwent the same handling as the two trained groups but

did not receive aversive training (Figure 21C; Figure 21D). TSWR and feeding post-tests were administered 24 h after training (Figure 21).

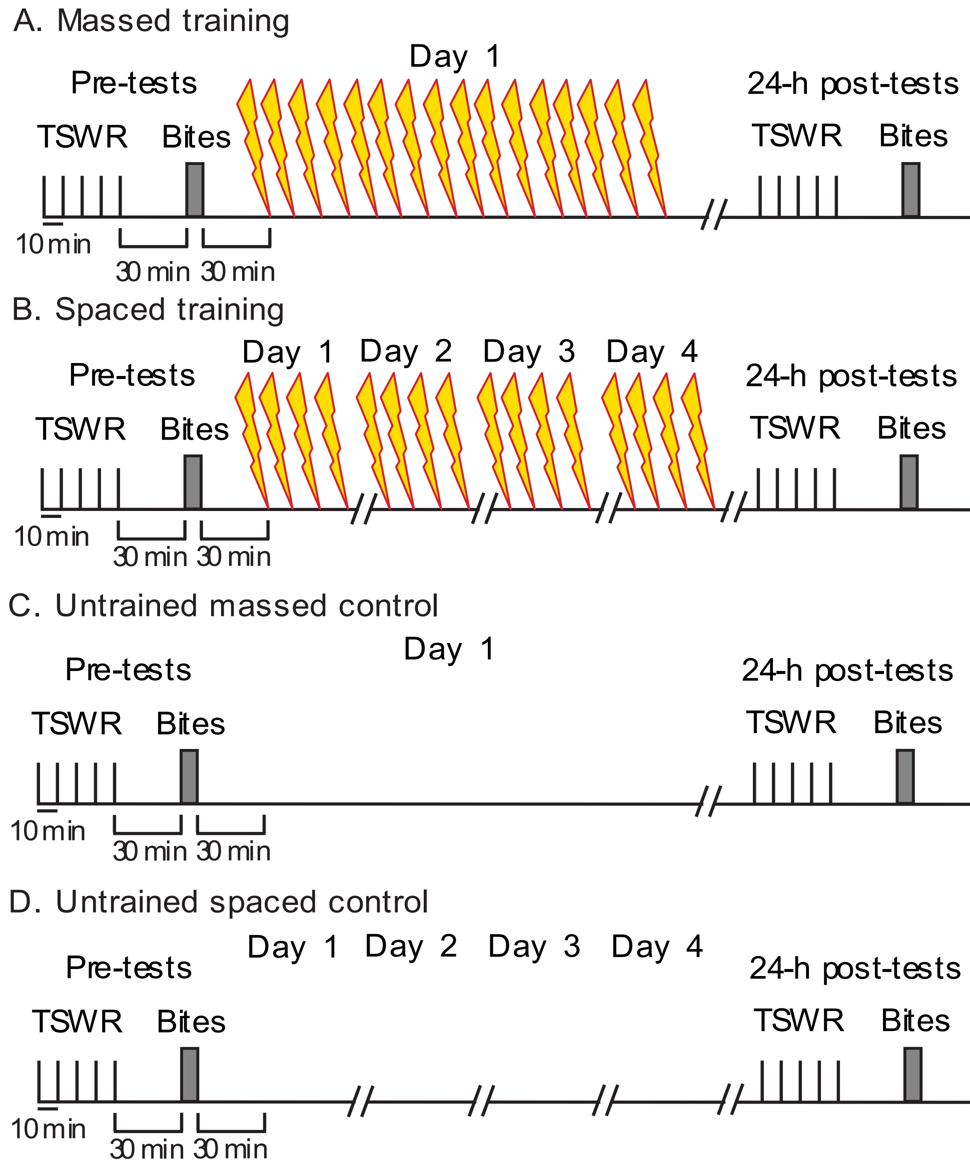

Figure 21: Protocols for massed and spaced training. A) Massed training protocol consists of 16 trials of training massed over 1 day. B) Spaced training protocol consists of 16 trials of training spaced over 4 days. C) Untrained control for massed protocol. D) Untrained control for spaced protocol. All post-tests were administered at 24 h (Wainwright et al., 2002 and Farruggella et al., 2019).

Methods – Objective 1b: Protocols for diurnal and nocturnal behavioral training

After the completion of TSWR and feeding pre-tests, animals were randomly selected to one of the following protocols: diurnal trained (D-T), diurnal untrained (D-UT), nocturnal trained (N-T), or nocturnal untrained (N-UT; Figure 22). Each group consisted of a 15-animal sample size. Training for both nocturnal and diurnal groups consisted of 4 trials, each made of 10-s trains of electrical stimuli (500-ms pulses, 1 Hz, 60-mA AC) spaced 30 min apart, delivered using a hand-held probe to the animal's body wall (Figure 17; Mac Leod et al., 2018). Previous work in *Aplysia* revealed that training conducted at ZT 9 (5 pm) induces maximal LTS (Fernandez et al., 2003). In addition, training at ZT 9 was found optimal for the activation of the molecular machinery necessary for LTM consolidation (Lyons et al., 2006). Conversely, training conducted at ZT 15 (11 pm) fails to induce LTS (Fernandez et al., 2003) and only marginally activates the biochemical cascade necessary for LTM formation (Lyons et al., 2006). Based on these findings, in this project diurnal training started at ZT 9 (Figure 22A), while nocturnal training started at ZT 15 (Figure 22B). The untrained control groups underwent the same handling as the training groups but did not receive aversive training (Figure 22C, Figure 22D). Pre-tests were conducted at different time points for diurnal (ZT 4) and nocturnal groups (ZT 11). Post-tests for all groups were conducted 18 h after the conclusion of training instead of the standard 24 h to allow for post-test behaviors to be measured during light hours given that they could not be reliably measured in the dark (Wainwright 2019, personal observation).

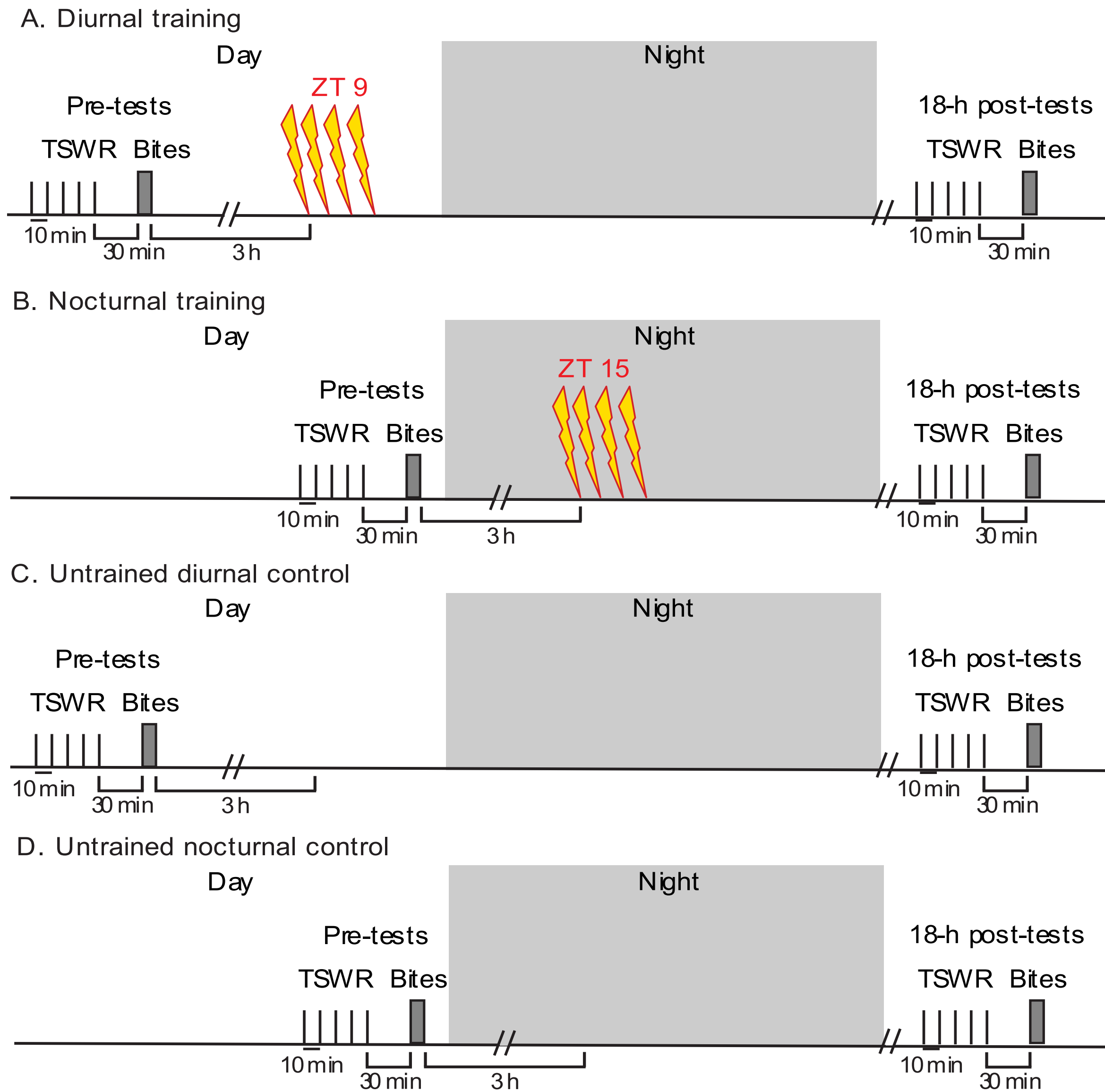

Figure 22: Protocols for diurnal and nocturnal training. A) Diurnal training protocol consists of 4 trials of training at ZT 9. B) Nocturnal training protocol consists of 4 trials of training at ZT 15. C) Untrained control for diurnal protocol. D) Untrained control for nocturnal protocol. All post-tests were be administered at 18-h after training (Modified from Fernandez et al., 2003 and Farruggella et al., 2019).

Analysis of behavioral data

The changes in feeding were analyzed as differences in bites, calculated as bites during pre-test subtracted from bites during post-test (Mac Leod et al., 2018, Farruggella et al., 2019). The change in TSWR duration was calculated as [post-test TSWR duration]/[pre-test TSWR duration] (Mac Leod et al., 2018, Farruggella et al., 2019). All data was represented as mean ± standard error of the mean.

In the massed training experiment **(Objective 1a)**, changes in TSWR duration and differences in bites were compared prior to aversive training/no training and then again at 24 h post-test, respectively. Massed training protocols differed greatly from spaced training in amount of handling, total days, and consequently were not suitable for multiple comparisons across the 4 groups. Thus, statistical analysis was instead performed by comparing M-T with M-UT groups and S-T with S-UT groups using Mann-Whitney U tests (Wainwright et al., 2002).

In the nocturnal training experiment (**Objective 1b**), changes in TSWR duration and differences in bites were compared prior to aversive training/no training and then again at 18 h post-test, respectively. Following the conclusion of behavioral testing, assessment of baseline bites revealed that nocturnal-trained animals had significantly higher pre-test bites compared to diurnal-trained animals (Figure 23; mean baseline bites; Diurnal: 20.833±1.268, n=30; Nocturnal: 25.700±1.722 bites, n=30; $p < 0.05$, U=311.500; Mann-Whitney U test). This unexpected difference in pre-test values can be attributed to pre-tests being conducted at different time points for diurnal (ZT 4) and nocturnal groups (ZT 11). Consequently, statistical analysis could not be

conducted across the 4 groups but was instead performed by comparing D-T with D-UT groups and N-T with N-UT groups using Mann-Whitney U tests.

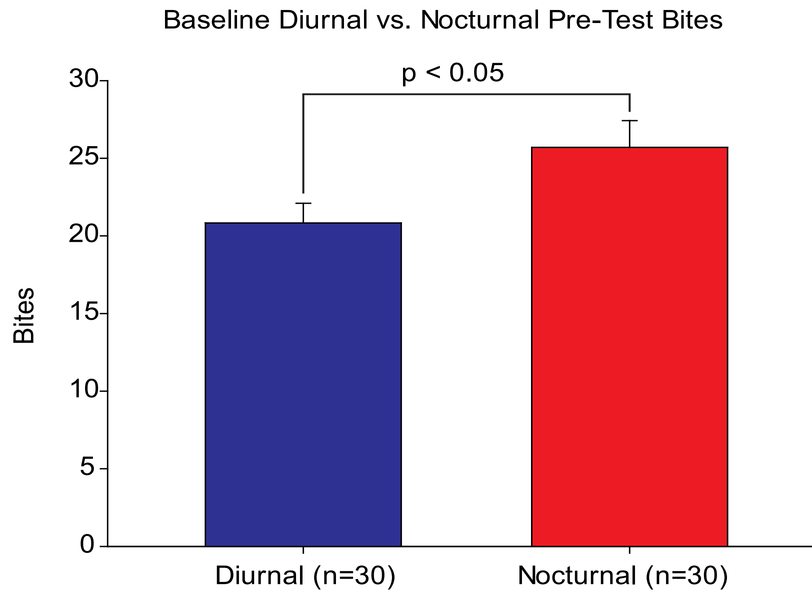

Figure 23: Baseline assessment of pre-test bites revealed a statically significant difference between diurnal and nocturnal groups.

For each experiment, estimated sample sizes were based on the effect size and standard error of similar studies from the Mozzachiodi lab and/or the literature. Sample size determination was guided by the use of the sample size calculator in SigmaPlot (Jandel Scientific), with α set at 0.05 and desired power set at 0.8. Projected sample size for **objective 1a**, which includes the 4-day spaced and the 1-day massed training, was set as n=10 for each group, whereas projected sample size for **objective 1b**, which includes the 1-day diurnal and the 1-day nocturnal training, was set at n=15 for each group. This difference between the projected sample sizes for each objective was set based on previous experiments that revealed that 4-day spaced training induces more robust LTS compared to 1-day training.

Methods – Objective 2: Cellular experiments

Animal dissection and tissue preparation for cellular experiments

An *in vitro* preparation was used to explore the necessity of transcription and translation in B51 LTDE (Figure 12; Weisz et al., 2017). Naive 48-h food deprived animals were dissected according to previously established protocols (Weisz et al., 2017, Farruggella et al., 2019). Once removed from the holding tank, a small piece of seaweed was presented to the animal to trigger a bite. This indicated that the animal was healthy, motivated to produce a bite, and could then be utilized for *in vitro* analysis. Animals were anesthetized by injecting a volume of isotonic $MgCl_2$ equal to 50% of the animal's mass into the hemocoel through the foot (Scholz and Byrne, 1987). After waiting several minutes or until the animal relaxed, forceps were used to touch the rhinophores or the tail to assess if the animal was responsive to tactile stimuli. If the animal was not responsive, then this confirmed that the animal was fully anesthetized. Once fully anesthetized, the animal was placed on a dissecting tray, ventral side up, and a longitudinal incision was made along the midline of the foot to expose the central nervous system. The incision was extended roughly one-third the length of the animal's body to the lips. The buccal, cerebral, and pleural-pedal ganglia were removed together with their interganglionic connectives to retain the critical components of the neural circuits controlling feeding and TSWR (Cleary et al., 1998, Weisz et al., 2017). One of the two buccal nerves 2,3 (B.n.2,3) were retained for identification of neuron B51 (Shields-Johnson et al., 2013, Weisz et al., 2017). Nerves P8 and P9 were also retained in the preparation for the delivery of *in vitro* aversive training (Figure 12; Weisz et al., 2017). All other nerves were cut short, and the ganglia were prepared according to the *in vitro* preparation (Figure 12). The isolated ganglia were transferred to a Sylgard-coated petri dish containing high-divalent ASW composed of 210 mM NaCl, 10 mM KCl, 145 mM $MgCl_2$, 20 mM $MgSO_4$, 33 mM $CaCl_2$

and 10 mM HEPES with pH of 7.5 (Figure 24; Weisz et al., 2017). The cerebral and pleural-pedal ganglia were pinned ventral side up and the pleural ganglia were desheathed on the ventral surface to expose the TSN cluster (Weisz et al., 2017). The buccal ganglia were flipped to expose the rostral side and were desheathed to access B51 (Weisz et al., 2017). In *Aplysia*, LTS and TSN LTIE are lateralized to the side of aversive training (Byrne and Hawkins, 2015), whereas LTFS and B51 LTDE are not lateralized (Acheampong et al., 2012). Consequently, either one of the B51 cells in the buccal ganglia were chosen for analysis regardless of whether they are ipsilateral or contralateral to the retained P8 and P9 nerves (Acheampong et al., 2012, Weisz et al., 2017). Conversely, only TSNs ipsilateral to the retained P8 and P9 nerves were utilized (Weisz et al., 2017). Bipolar extracellular stimulating electrodes were placed along P8, P9, and B.n.2,3 nerves and isolated from the bath with Vaseline (Mozzachiodi et al., 2003, Weisz et al., 2017). The high-divalent ASW was replaced by normal ASW, and the preparation was allowed to rest for 30 min

Figure 24: Isolated ganglia placed in Sylgard-coated petri dish. (Photo credit: Edgar De La Garza)

(Weisz et al., 2017). The recording chamber was maintained at 15˚C throughout the electrophysiological recordings with a feedback-controlled cooling device (Model BTC-100/BTC-S, Bioscience Tools, CA; Weisz et al., 2017).

B51 intracellular recordings

Two-electrode current-clamp was used for intracellular recordings from B51. Since the B51 cell body is relatively large, two electrodes can consistently be used for recording and current stimulation, respectively (Nargeot et al., 1999b). B51 was identified by its relative size and position within the buccal ganglia and by the occurrence of its all-or-nothing plateau potentials (Figure 25; Weisz et al., 2017). If B51 did not fire spontaneously or in response to a brief intracellular depolarizing current, then B.n.2,3 was briefly simulated (1-2 s of 10 V, 0.5-ms pulse at 4 Hz) and the response of the cell was used to aid in identifying B51 (Weisz et al., 2017). B51 resting membrane potential (V_m) was determined from the recording electrode readout after the cell was allowed to rest for 5 min. Resting membrane potential (V_m) measures the difference in electrical charge across the membrane, which also helps indicate the baseline health of the cell. Cells were included in the study only if they display a resting membrane potential of at least -45 mV (Weisz et al., 2017). Following B51 V_m measurement, B51 was clamped -60 mV because each cell may have different individual resting potentials but clamping the resting membrane potential provided a baseline for all cells to have the same starting membrane potential (Weisz et al., 2017). Once B51 was clamped at -60 mV, the input resistance (R_{in}) was determined by injecting 5 nA of hyperpolarizing current for 5 secs, measuring the change in voltage from the baseline before pulse to just before pulse ends, and solving Ohm's Law ($I = V/R$) for resistance (Figure 25A). R_{in} measures the number of open channels at rest. Excitability of B51 was measured by the neuron's burst threshold. Burst threshold (BT) was determined by delivering a series of 5 s depolarizing

current pulses, beginning at 5-nA intensity, and increasing in intensity in 1-nA increments, at 10 s intervals, until the cell fired burst activity that outlasted the injected current (Figure 25B; Nargeot et al.,1999a; Mozzachiodi et al., 2008; Weisz et al., 2017). B51 V_m, R_{in}, and BT are intrinsic neuronal properties of the cell that can be modified by learning (i.e., neuronal plasticity; Mozzachiodi and Byrne, 2010).

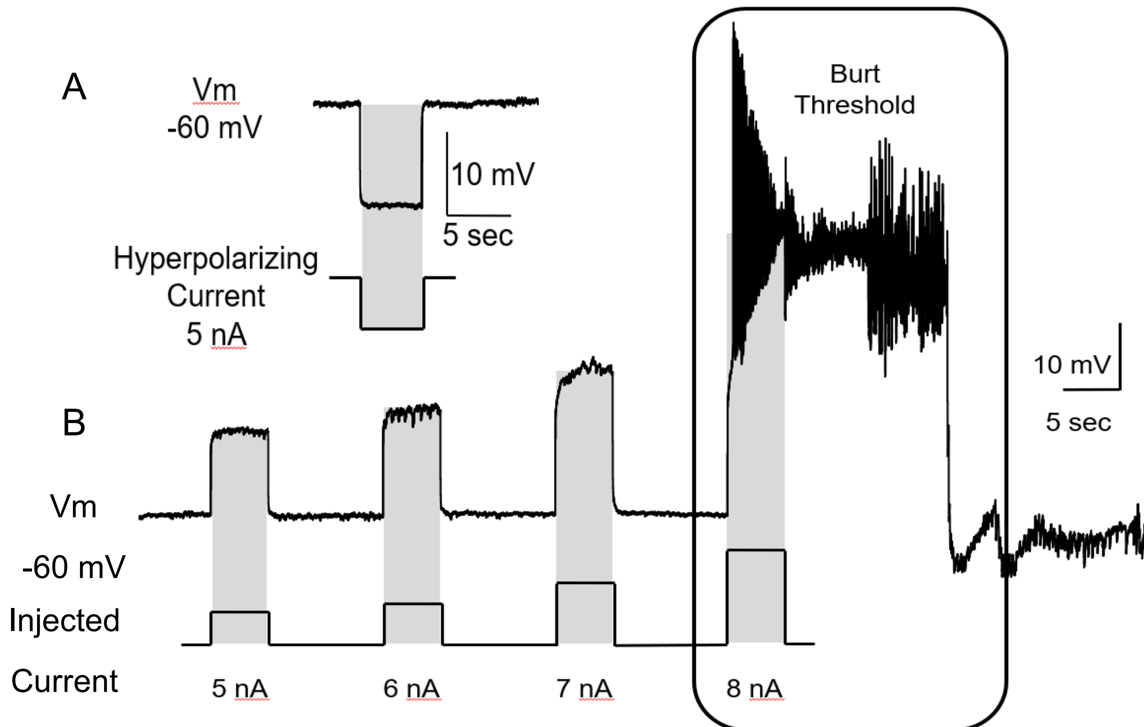

Figure 25: B51 measurements. A) Input resistance was measured by dividing the voltage drop elicited by a 5-s, 5-nA hyperpolarizing current by 5 s. B) Excitability of B51 was measured by determining the burst threshold (BT). BT was measured by delivering 5-s pulses of depolarizing current, starting at 5 nA and increasing by 1 nA until the cell fired its all-or-nothing plateau potential. In the example illustrated, BT was 8 nA.

TSN intracellular recordings

Because TSNs cell bodies are smaller than B51, the two-electrode current clamp method cannot consistently be used without damaging the cell. Therefore, one electrode was used for both recording and current stimulation instead (Cleary et al., 1998, Weisz et al., 2017). The TSNs were identified by their relative size and their position in the pleural ganglia, and the occurrence of

action potentials in response to a single 3-msec stimulus delivered to nerve P9 (Cleary et al., 1998, Weisz et al., 2017). TSN resting membrane potential (Vm) was determined from the recording/stimulating electrode readout after the cell was allowed to rest for 5 min. TSN cells were only be included in the study if they display a resting membrane potential of at least – 35 mV (Weisz et al., 2017). Following the V_m measurement, TSNs were clamped at – 45 mV for consistency within the study (Weisz et al., 2017). Resting membrane potential of TSNs were clamped at -45 mV because each cell may have different individual resting potentials but clamping the resting membrane potential provided a baseline for all cells to have the same starting membrane potential. Excitability of TSNs was then assessed by counting the number of action potential spikes generated from a 1 sec, 2 nA depolarizing current (Figure 26; Cleary et al., 1998, Weisz et al., 2017). TSN resting membrane potential (V_m) and the number of spikes are intrinsic neuronal properties of the cell that can be modified by learning (i.e., neuronal plasticity; Weisz et al., 2017, Mozzachiodi and Byrne, 2010).

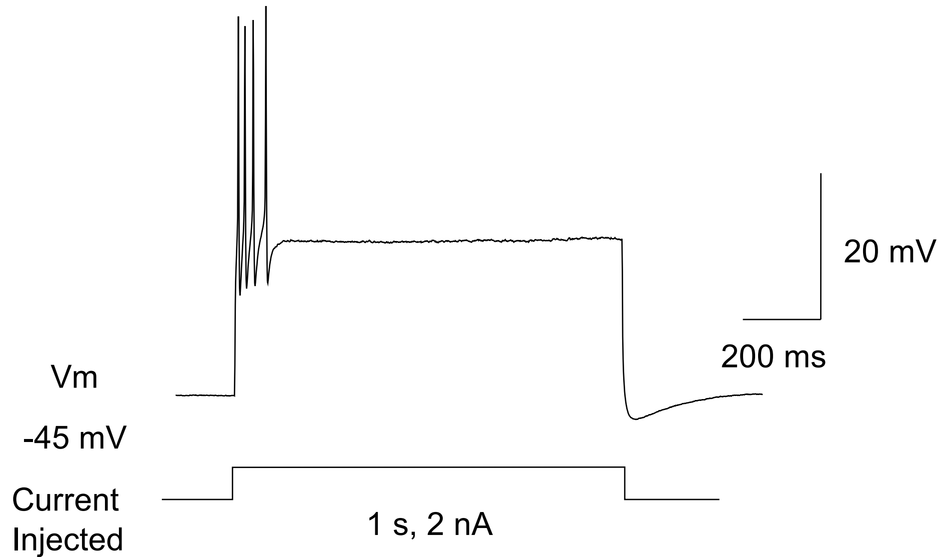

Figure 26: TSN excitability was measured by injecting a 1-sec, 2 nA pulse of depolarizing current and counting the number of action potentials evoked (4 action potentials in example above).

Incubations of selective transcription and translation inhibitors

The prepared ganglia were incubated in either ASW+0.1% DMSO, translation inhibitor ANI (20 µM), or transcription inhibitor ACT-D (50 µg/ml) prior to pre-tests (Castellucci et al., 1986, Montarolo et al., 1986). DMSO (0.1%) was used as a vehicle for all incubations (e.g., Farruggella et al., 2019). ACT-D was initially bath-applied for 45 min and was exchanged with ASW 15 min prior to the beginning of intracellular recordings (Figure 28A; Montarolo et al., 1986). ANI was bath-applied from 60 min prior to initial recording until 60 min after the end of the *in vitro* training (Figure 28B; Schacher et al., 1988). The proposed concentrations and durations of bath-applications of the blockers were chosen from previous studies because they successfully inhibit transcription and translation in *Aplysia* and block forms of learning-induced long-term cellular plasticity in the TSWR circuit (Montarolo et al., 1986, Schacher et al., 1988).

Testing and training procedures

TSN and B51 membrane properties were initially measured prior to *in vitro* training (pre-tests. *In vitro* training was comprised of 4 trials, each consisting of 10 s trains of P8/P9 electrical stimulation, spaced 30 min apart (Figure 28; Zhang et al., 1994, Weisz et al., 2017; Farruggella et al. 2019). Untrained controls consisted of preparations that did not receive any nerve stimulation (Weisz et al., 2017). Following completion of pre-test measurements, cells surrounding identified TSN were injected with fast-green dye, and then annotated pictures were taken of both B51 and identified TSN using an AmScope (AmScope Digital Microscope USB Camera MU900, 9-megapixel resolution) to aid in post-test cell identification (Figure 27). Surrounding cells were injected with fast green dye instead of the identified cell to avoid altering intrinsic properties of the target TSN used for experimental analysis (Figure 27).

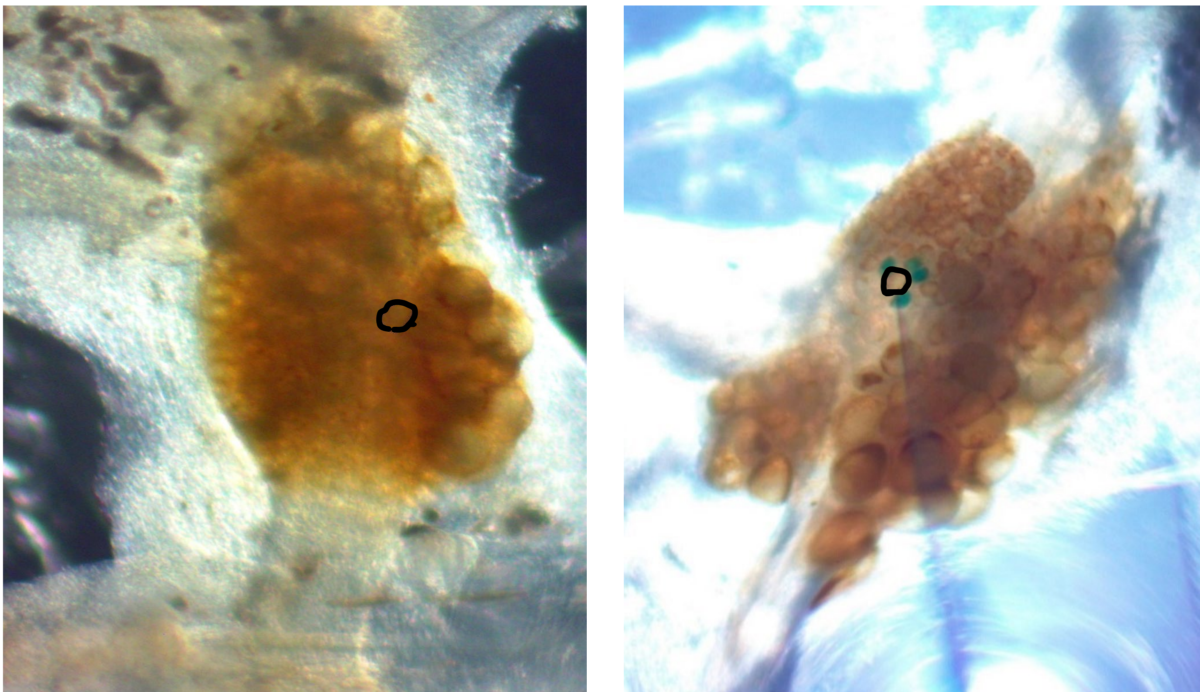

Figure 27: Annotated B51 (left; buccal ganglia) and TSN (right; pleural ganglia) used for post-test cell identification. Cells were annotated using Windows Paint software by circling the perimeter of their cell bodies.

After the end of training/no training, the isolated ganglia were placed in L-15 culture medium and stored at 15°C for 23.5 h. On the next day, ganglia were rinsed in normal ASW and was allowed to rest for 30 min before the post-test (Weisz et al., 2017). TSN and B51 membrane properties were measured again 24 h after training (post-tests; Figure 28; Weisz et al., 2017). Six groups were used: trained/vehicle, trained/ANI, trained/ACT-D, untrained/vehicle, untrained/ANI, and untrained/ACT-D.

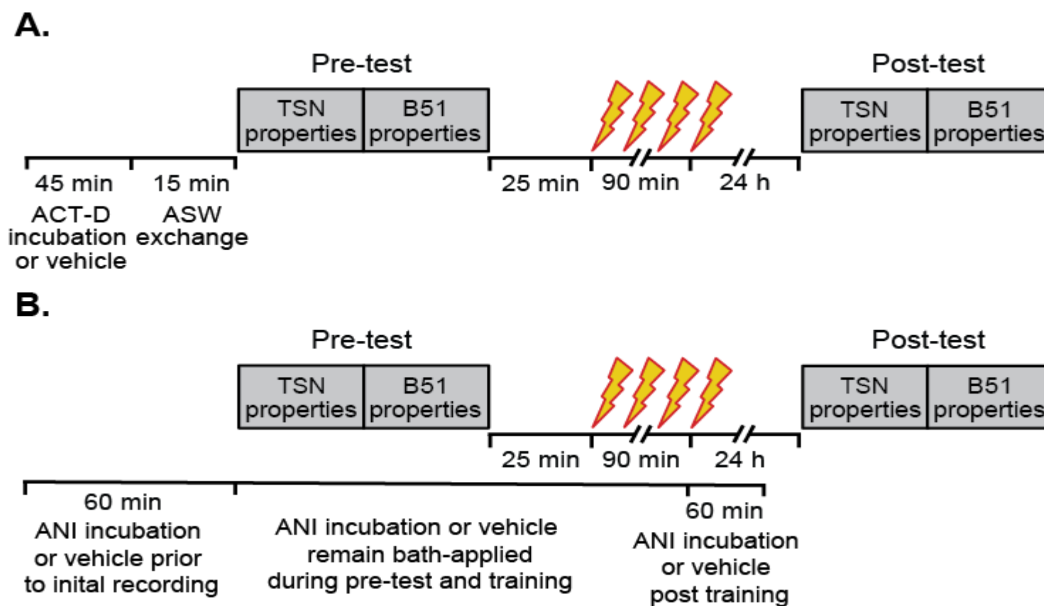

Figure 28: Protocols for cellular methods with inhibitors, incubation, pre-test, training, and 24 h post-tests for the experiments using treatment with either ACT-D (A) or ANI (B; Modified from Farruggella et al. 2019).

Analysis of cellular data

For each measurement from TSNs (V_m, number of spikes) and B51 (V_m, R_{in}, BT), the percent change was calculated as [(post-pre/pre) x 100] to assess modifications due to training and/or treatment (Weisz et al., 2017). TSN and B51 measurements were compared among the six groups using the Kruskal-Wallis test to determine overall statistical significance followed by

Dunn's *post hoc* comparison to isolate sources of significance (Farruggella et al., 2019). Projected sample size was based on the effect size and standard error of similar studies from the Mozzachiodi lab and/or the literature. Sample size determination was guided by the use of the sample size calculator in SigmaPlot (Jandel Scientific), with α set at 0.05 and desired power set at 0.8. Projected sample size for **objective 2** was set as n=15 for each group. Electrophysiology measurements from both B51 or TSNs could not always be collected from the same preparation because 1) the inability to find B51 or TSNs, 2) the inability to maintain recordings throughout the entire experiment, 3) the cell's inability to fire in response to injected current or 4) damage/loss of cell during electrode implantation. This discrepancy in the ability to collect electrophysiology measurements from every preparation hindered my ability to meet the projected sample size of n=15 in each group, and thus sample sizes are currently unequal.

CHAPTER IV: RESULTS

Results: Objective 1a

Massed training induces LTFS, but not LTS

The goal of **objective 1a** was to explore the effects of massed training on LTFS. Results indicated that massed training induced LTFS in the absence of LTS at 24 h. M-T animals expressed LTFS compared to M-UT animals (Figure 29A; mean difference in bites; M-T: -21.500±3.367 bites, n=10; M-UT: -4.200±2.380 bites, n=10; $p < 0.05$, U=8.000; Mann-Whitney U test). Similarly, spaced training (S-T), which served as the positive control for training-induced behavioral changes, also induced LTFS compared to the S-UT group (Figure 29B; mean difference in bites; S-T: -16.700±2.093 bites, n=10; S-UT: 3.500±2.442 bites, n=10; $p < 0.05$, U=1.500; Mann-Whitney U test).

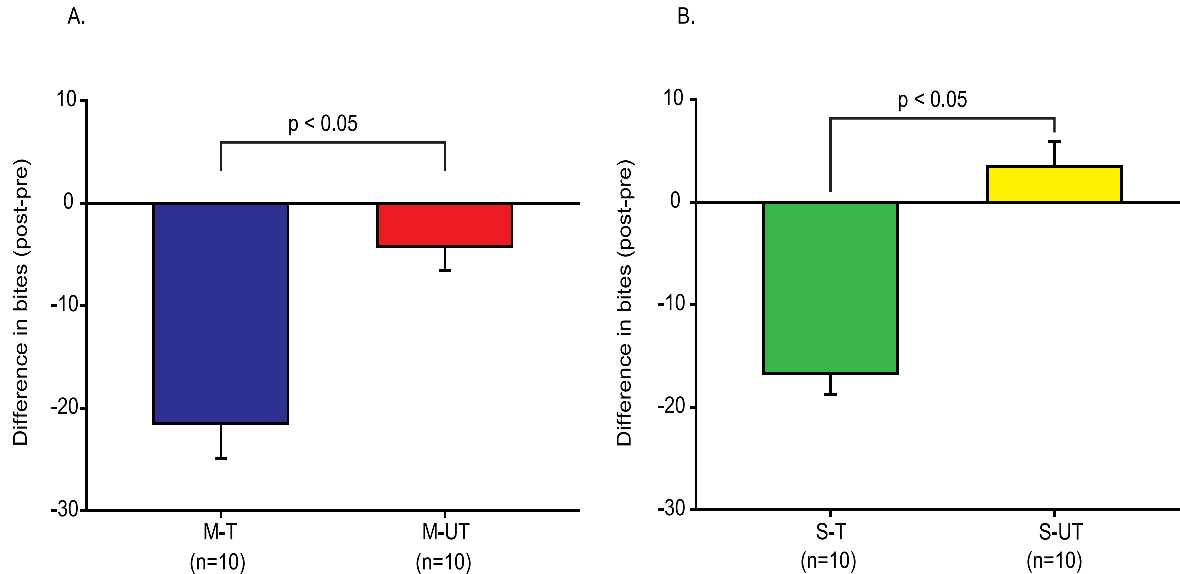

Figure 29: Results showing that both massed training (M-T) and spaced training (S-T) induce LTFS at 24 h.

However, **objective 1a** results also revealed that massed training (M-T) did not induce LTS (Figure 30A; mean change in TSWR; M-T: 1.029±0.053 secs, n=10; M-UT: 1.144±0.079 secs, n=10; *p*=0.385, *U*=38.000; Mann-Whitney U test). S-T animals expressed LTS compared to S-UT animals, which successfully replicated pervious results (Figure 30B; mean change in TSWR; S-T: 1.564±0.191 secs, n=10; S-UT: 0.999±00.094 secs, n=10; $p < 0.05$, *U*=22.000; Mann-Whitney U test).

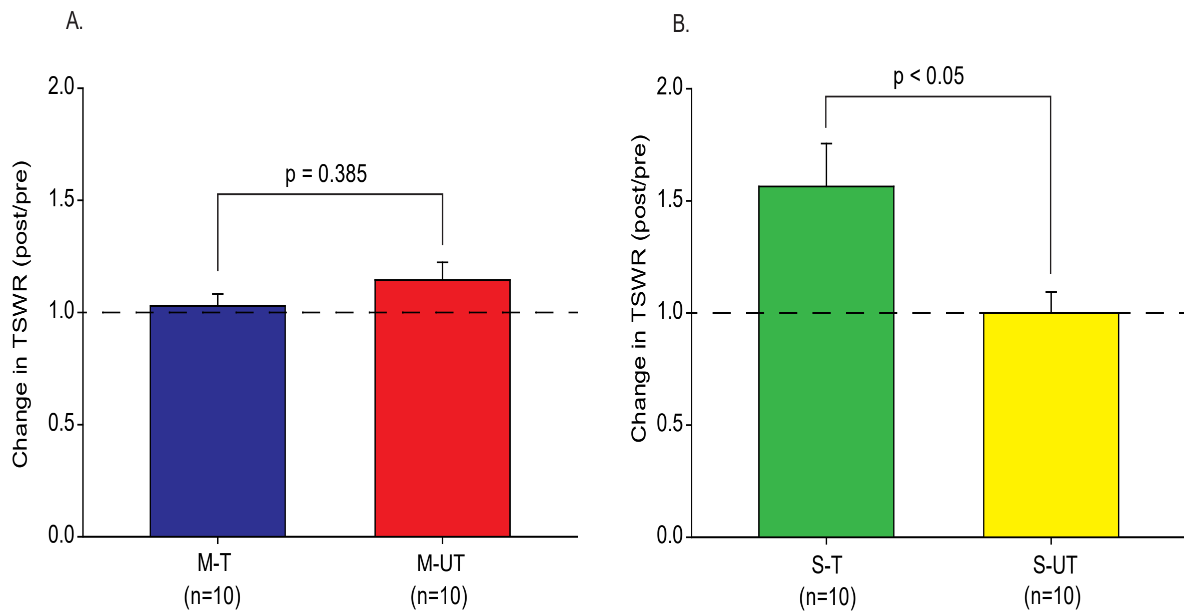

Figure 30: Results indicating that massed training (M-T, A) does not induce LTS. Spaced training (S-T, B) results successfully replicated learning induced TSWR behavioral changes.

Results: Objective 1b

Nocturnal training does not induce LTFS or LTS

The goal of **objective 1b** was to determine the effects of nocturnal training on LTFS. Specifically, this objective explored if the circadian rhythm modulates feeding suppression similar to how it modulates LTS. Results revealed that nocturnal training (N-T) does not induce LTFS or LTS at 18 h indicating that the circadian rhythm modulates both appetitive and defensive behaviors. Diurnal trained (D-T) animals expressed LTFS at 18 h, replicating previously established results (Figure 31A; mean difference in bites; D-T: -7.667±2.620 bites, n=15; D-UT: 1.267±0.938 bites, n=15; $p < 0.05$, U=47.000; Mann-Whitney U test). Conversely, no statistical difference was found comparing N-T group to the N-UT group (Figure 31B; mean difference in bites; N-T: -2.000±2.234 bites, n=15; N-UT: -4.267±1.700 bites, n=15; p=0.406, U=92.000; Mann-Whitney U test).

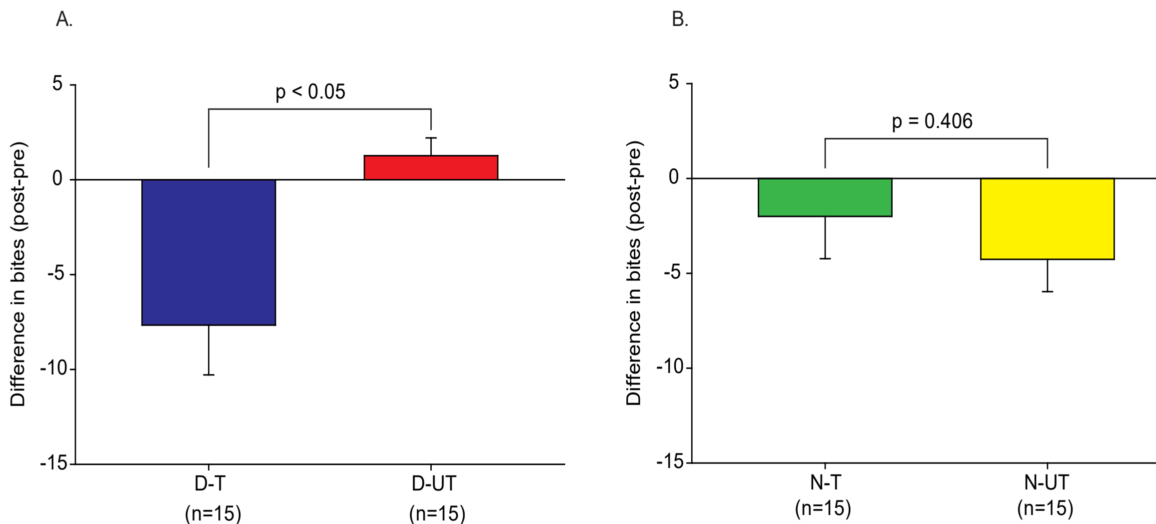

Figure 31: Results showing LTFS in the D-T group compared to D-UT group. However, N-T did not induce LTFS compared to the N-UT group at 18 h.

Objective 1b results also indicate that diurnal trained (D-T) animals expressed expected increase in TSWR duration compared to untrained animals (D-UT; Figure 32A; mean change in TSWR; D-T: 1.530±0.195 secs, n=15; D-UT: 1.116±0.046 secs, n=15; $p < 0.05$, U=53.000; Mann-Whitney U test). However, similar to LTFS, nocturnal training did not induce LTS comparing the N-T group to the N-UT group (Figure 32B; mean change in TSWR; N-T: 1.055±0.037 secs, n=15; N-UT: 1.008±0.037 secs, n=15; p=0.709, U=103.000; Mann-Whitney U test)

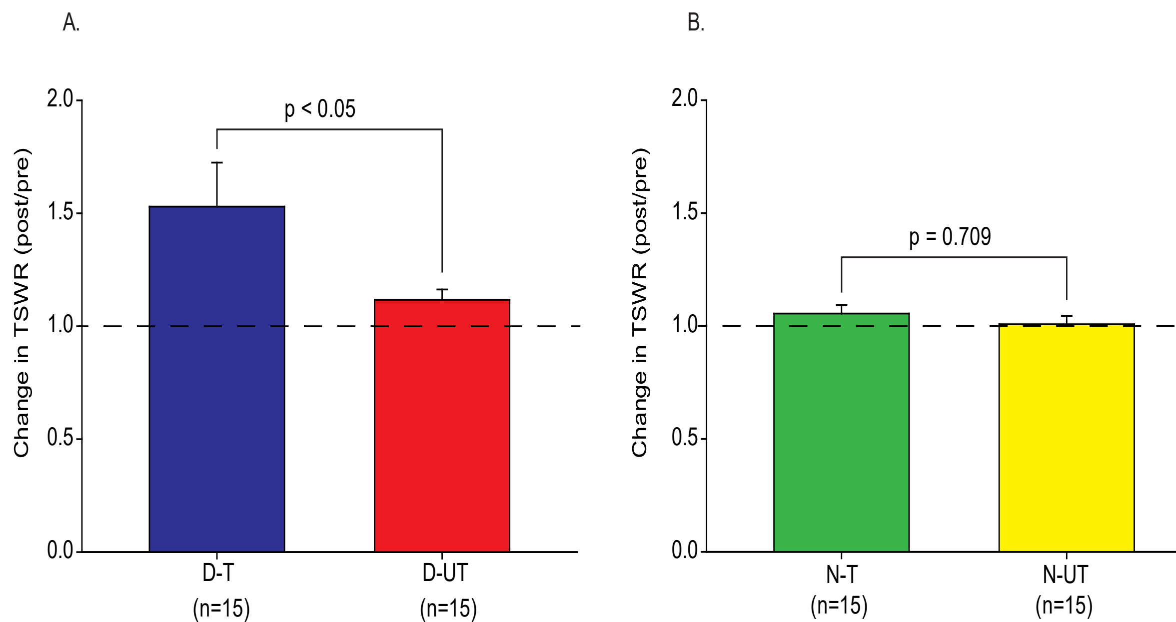

Figure 32: Results showing LTS comparing the D-T to D-UT group. However, N-T did not induce LTS at 18 h.

Results: Objective 2

Analysis of the contributions of transcription and translation to B51 LTDE

Objective 2 investigated whether long-term changes in B51 and TSN excitability is transcription and/or translation dependent. Training-induced TSN LTIE served as a positive control for the effectiveness of the transcription and translation blockers (Montarolo et al., 1986). As the projected sample size of n=15 was not met in each group, results will report suggested trends but not finalized conclusions. Thus, current TSN results thus far suggest a trend that both ACT-D and ANI effectively inhibit TSN LTIE, which would replicate previously established results if statistical significance were achieved (Montarolo et al., 1986). A Kruskal-Wallis determined overall statistical significance in TSN LTIE among the six groups (Figure 33; H= 18.805 with 5 degrees of freedom; $p < 0.05$; Kruskal-Wallis test). Dunn's *post hoc* comparison test revealed a significant difference between the T-V and T-ACT-D groups ($p < 0.05$; q=3.852), and

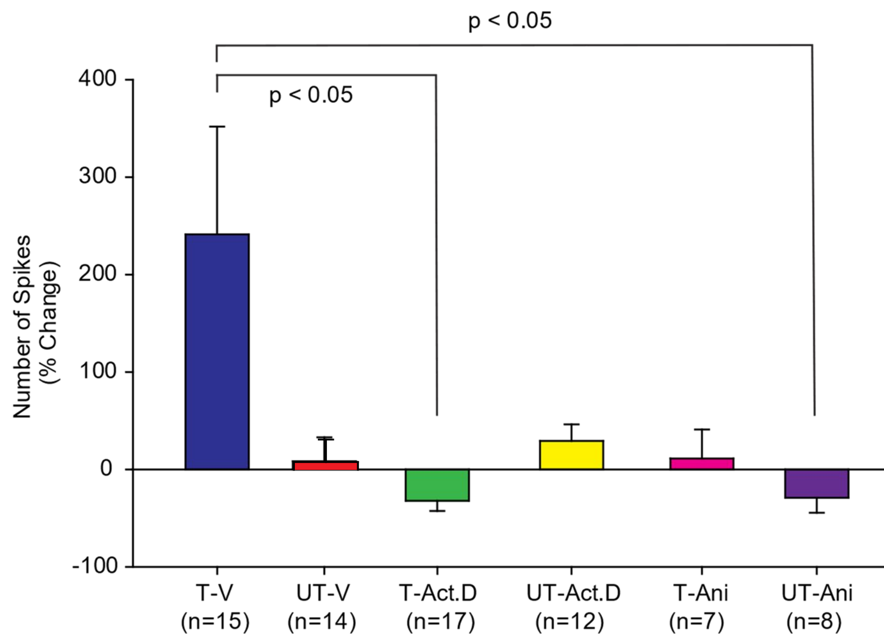

Figure 33: Current results indicate a trend toward ACT-D and ANI inhibiting TSN learning induced plasticity compared to the control T-V group.

between the T-V and UT-Ani groups (Figure 33; $p < 0.05$; q=2.985). Although statistical significance has not been achieved yet between the T-V and UT-V groups, LTIE in the T-ANI group was lower compared to the T-V group, which suggests that ANI is effective in blocking the expression of LTIE similar to ACT-D. Based on current trends, we could expect a statistical significance between the T-V and UT-V groups, and then TSN LTIE to be inhibited by ANI and ACT-D as this experiment is finalized. However, as I was unable to meet the **objective 2** projected sample size of n=15 for each group, the experiment will be ongoing to fulfill the projected sample size.

No statistical difference was found comparing the resting membrane potential (Vm) across the six groups (Figure 34; H= 9.342 with 5 degrees of freedom; *p*=0.096; Kruskal-Wallis test).

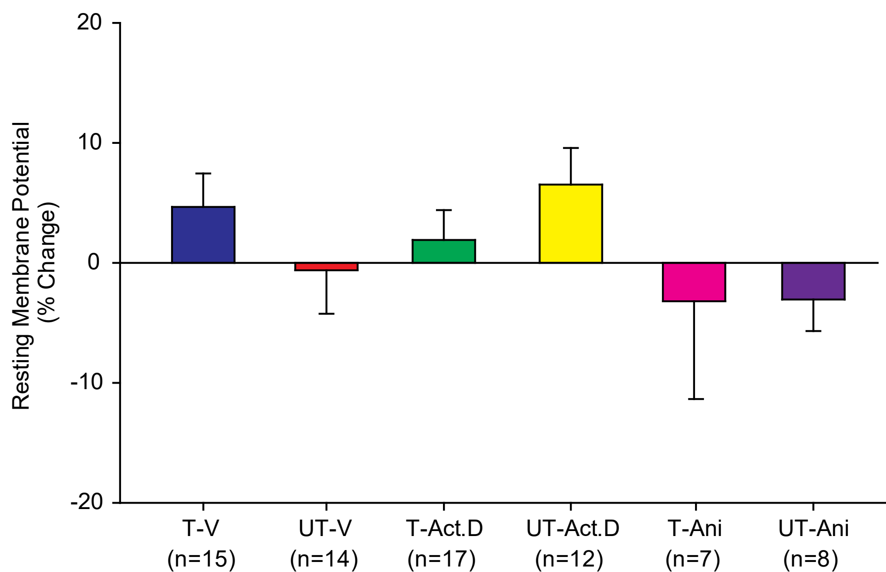

Figure 34: Results indicating no statistical difference found comparing the TSN V_m across the six groups.

Similar to TSN LTIE, current B51 results suggest a trend for B51 LTDE to be inhibited by ACT-D and ANI. Although a Kruskal-Wallis test determined no overall statistical significance among the six groups (p = 0.146), current results show a trend for B51 LTDE to occur in the T-V group compared to the UT-V group, but not in the and T-ACT-D group or in the T-ANI group (Figure 35; H= 8.185 with 5 degrees of freedom; p=0.1461; Kruskal-Wallis test). If statistical significance is achieved, our findings will indicate that both ACT-D and ANI inhibit B51 LTDE in a manner similar to TSN LTIE.

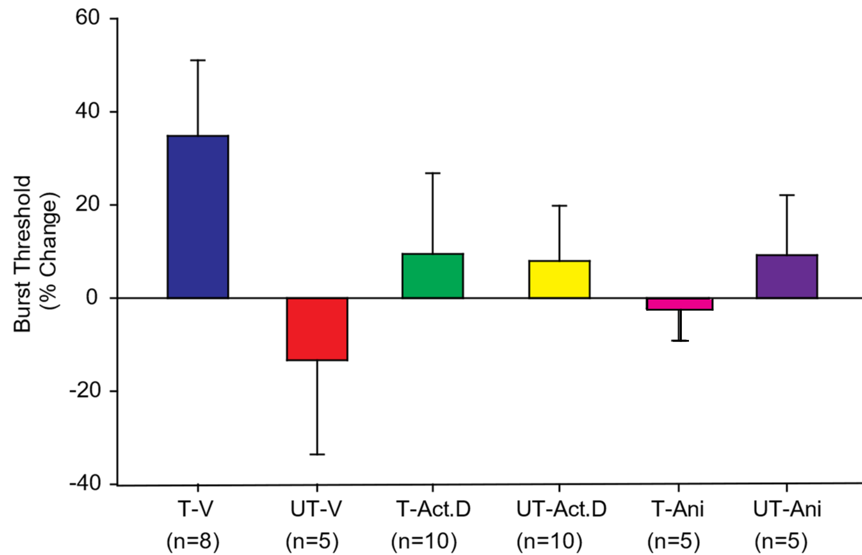

Figure 35: Current results indicate a trend toward ACT-D and ANI inhibiting B51 learning induced plasticity compared to the control T-V group

No statistical difference was found comparing the input resistance (R_{in}) across the six groups (Figure 36; H= 7.845 with 5 degrees of freedom; p=0.165; Kruskal-Wallis test). Although, trends are showing a possible R_{in} increase in the T-ACT.D, which suggests that the number of open ion channels may be increased in this experimental group. This increase in B51 R_{in} would

lead to the increased expression of buccal motor programs (BMPs) that generate bites by the animal (Nargeot et al., 1999a, Farruggella et al., 2019).

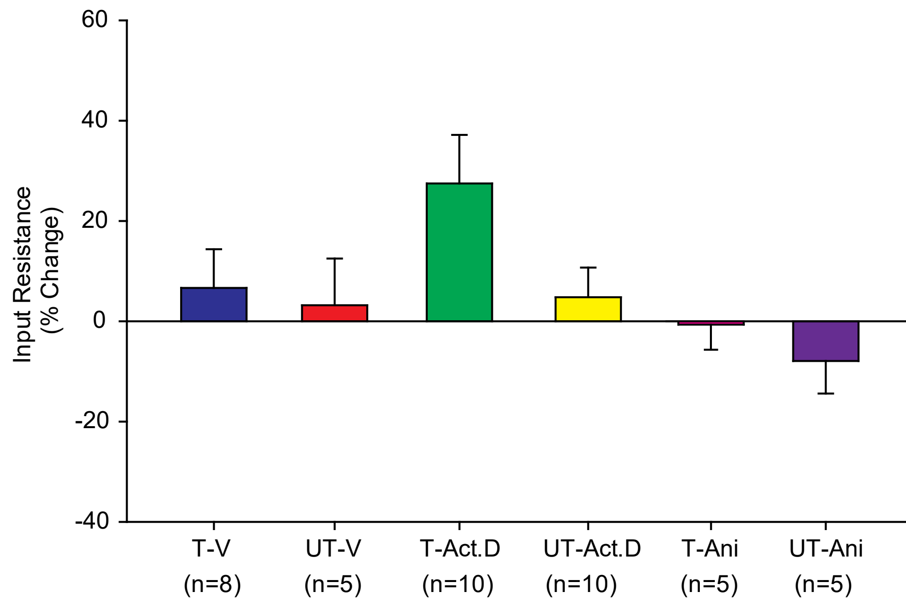

Figure 36: Results indicating no statistical difference found comparing B51 input resistance (R_{in}) across the six groups.

No statistical difference was found comparing the resting membrane potential (V_m) across the six groups (Figure 37; H= 1.337 with 5 degrees of freedom; p=0.931; Kruskal-Wallis test).

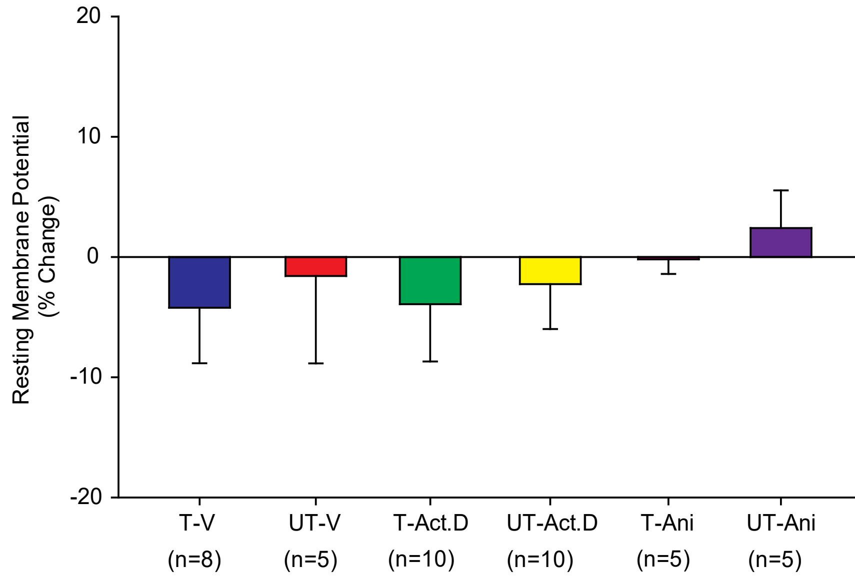

Figure 37: Results indicating no statistical difference found comparing B51 resting membrane potentials (V_m) across the six groups.

CHAPTER V: DISCUSSION

Massed training induces LTFS in the absence of LTS

Results from **objective 1a** revealed that massed training leads to the expression of feeding suppression in the absence of sensitization. The uncoupling of these two behavioral modifications is similar to previous findings reported in food deprived *Aplysia*. Animals that were 14-day food deprived expressed LTFS even in the absence of LTS following long-term aversive training protocols (Mac Leod et al., 2018). This uncoupling of LTFS from LTS in food deprived animals suggested that these two behavioral modifications may be acting independently of one another. Thus, results from **objective 1a** provide further evidence of protocols that uncouple LTFS from LTS. Specifically, results from **objective 1a** showed that massed training is not conducive for LTS. Although not reported in this thesis, post-tests for LTS and LTFS were also conducted 48 and 72 h after the conclusion of training to account for any delayed responses. No statistical significance was found following spaced, massed, diurnal, or nocturnal training after 24 h post-test.

Extensive research into the defensive neural circuit of *Aplysia* has shown that LTS is mediated, at least in part, by 5-HT (Glanzman et al., 1989; Levenson et al., 1999). Following exposure to aversive stimuli, 5-HT is released into the neuropil and into the hemolymph of the animal (Glanzmann et al., 1989; Levenson et al., 1999). 5-HT is known to induce TSN increased excitability as well as an increase in synaptic connection between the TSNs and motor neurons that produce the TSWR (Byrne and Hawkins, 2015). These intrinsic and synaptic properties of the TSNs and MNs sustain LTS (Byrne and Hawkins, 2015). Previous research into the long-term effects of 5-HT on LTS have shown that 5-HT levels released in the hemolymph remain significantly elevated for at least 24 h following a 1-day protocol consisting of 4 trials of aversive training (Levenson et al., 1999). This elevation of 5-HT levels helps sustain long-term behavioral

sensitization (Levenson et al., 1999). Although 5-HT levels were not measured in this study, we expect that 5-HT levels were significantly elevated following spaced training, which helped sustain the observed LTS. The spaced training protocol utilized in this study, which consisted of 16 trials of aversive training spaced over 4 days, paralleled the previous protocols that indicated that 5-HT levels remained elevated for at least 24 h following a 1 day 4 trial protocol of aversive training (Levenson et al., 1999). The current spaced training protocol in this study utilized 16 trials of 10 electrical shocks to the animal's body wall delivered over 4 days of training, which induced LTS lasting for at least 24. The main difference between the previously established methods in Levenson et al., 1999 and the spaced training utilized in this experiment was the total amount of training. Spaced training utilized the same pattern of electrical stimulation delivered during aversive training, but the total amount of training was extended from 4 trials over 1 day to 4 trials over 4 days of training. Thus, we expect 5-HT levels to be elevated following current spaced training protocols based on the similarities between the aversive training methods utilized in Levenson et al., 1999 and our current methods. However, in response to 16 trials of massed training in our current study, LTS was not expressed at 24 h, which suggests that other mechanisms may be counteracting the effect of 5-HT. Similar to 5-HT released during 4 trials of aversive stimuli, we expect that 5-HT is being released during massed training based on the similarities in the pattern of the electrical stimulation used during aversive training. The massed training protocol utilized in this study directly resembled the pattern of aversive training utilized in Levenson et al., 1999 for the first 4 trials of training. However, our massed training further prolonged the 1-day training from 4 trials to a total of 16 trials delivered over 7.5 h. Thus, we expect that 5-HT levels remain significantly elevated for at least 24 h following massed training based on previous research

detailed above. However, the effects of elevated 5-HT levels are no longer observed at 24 h following the massed training protocol.

Therefore, a possible hypothesis as to why the effects of 5-HT are no longer observed following massed training is the balancing effects of high levels of 5-HT counteracted by an inhibitory process. For instance, the neuropeptide Phe-Met-Arg-Phe-NH_2 (FMRFamide) is known to induce long-term depression (LTD) in the sensorimotor synapse of *Aplysia* (Fioravante et al., 2006). Massed training could be facilitating the accumulation of FMRFamide, which may counteract 5-HT dependent mechanisms by the activation of p38 mitogen-activated protein kinase (MAPK) and the transcriptional repressor phospho-CREB2. This accumulation of FMRFamide may override the LTFS normally induced by 5-HT (Figure 38; Fioravante et al., 2006). Thus, FMRFamide may inhibit LTS formation through the regulation of the CREB2 and MAPK cascading pathways (Figure 38; Fioravante et al., 2006). FMRFamide in *Aplysia* may act as a memory-suppressor neuromodulator by maintaining basal levels of p38 MAPK activation, CREB2 phosphorylation, and repression of genes responsible for LTS. Memory suppression neuromodulator mechanisms, such as FMRFamide, are known to constrain synaptic strengthening, preventing memory formation (Fioravante et al., 2006). Future directions into the interaction of 5-HT and inhibitory processes such as the activation of FMRFamide, which could counteract 5-HT, need to be explored to uncover why massed training is not conducive for LTS.

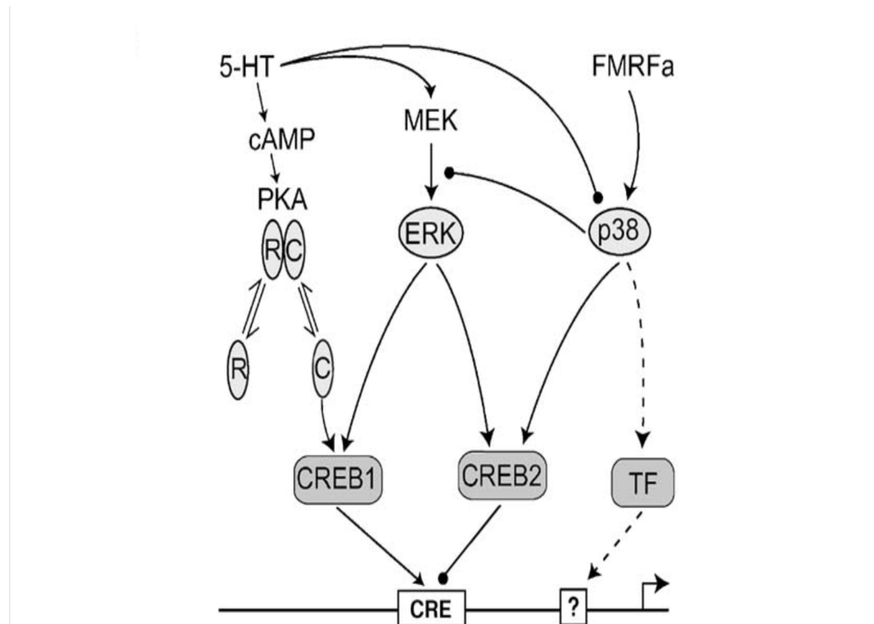

Figure 38: Model of competing effects of FMRFamide on 5-HT, kinase activation, transcription factor phosphorylation, and gene expression (Fioravante et al., 2006)

In response to fear, *Aplysia* is known to budget its defensive and appetitive behaviors by generally increasing defensives while also decreasing feeding motivation. However, as discussed above, these behavioral modifications can be decoupled from one another. Interestingly, results from **objective 1a** revealed that massed training was successful in inducing LTFS. Despite the lack of increased defenses, massed training decreases the animal's motivation to feed even in the presence of food. One possible explanation for this unexpected reconfiguration of the balance between defensive and appetitive responses may be the conservation of energy by the animal considering the nutrients gained versus the energy expenditure of feeding (Gillette et al., 2000; Mac Leod et al., 2018).

Previous research into the feeding neural circuit of *Aplysia* has indicated that, unlike LTS, 5-HT does not contribute to feeding suppression or B51 decreased excitability (Shields-Johnson et al., 2013, Weisz et al. 2017). LTFS is mediated at least in part by the gaseous neurotransmitter

nitric oxide (NO), which is known to be required for B51 LTDE (Farruggella et al., 2019). NO is synthesized by L-arginine, which like tryptophan, is obtained from the animal's diet. Similar to the release of 5-HT, which mediates LTS, NO is released by the animal in response to aversive training protocols. NO is widely used across different organisms for LTM formation and retention. NO has different molecular properties to more conventional neurotransmitters, such as 5-HT, which may allow for the continual release of NO in *Aplysia* (Susswein et al., 2004). For instance, unlike other neurotransmitters that are synthesized well before release and stored in lipid synaptic vesicles, when NO is used as a neurotransmitter, it is synthesized immediately before use (Susswein et al., 2004). In *Aplysia*, NO synthesis is triggered by an increased intracellular Ca^{2+} concentration, which activates the enzyme nitric oxide synthase (NOS; Bredt and Snyder 1992; Susswein et al., 2004). NO plays an extremely variable but vital role in LTM formation. For instance, sometimes NO plays a role in changes associated with initial learning or STM (Short Term Memory), such as short-term feeding suppression in *Aplysia* (Susswein et al., 2004; Farruggella et al., 2019). NO also mediates the expression of LTM such as the observed changes in feeding in this study. NO has been shown to sometimes be restricted to the time period or pattern of training (Susswein et al., 2004). Similar to current results from this study in *Aplysia*, previous research in the honeybee, *Apis mellifera,* classical conditioning indicate that NO is required for LTM following spaced training (Müller 1996; Susswein et al., 2004). The inhibition of NO synthase impairs LTM formation following classical conditioning in the honeybee (Müller 1996). Long-term changes in both excitatory and inhibitory synapses are commonly modulated by changes in neurotransmitter properties that can persist for hours (Castillo, 2012; Yang and Calakos, 2013; Monday et al., 2018). Consequently, differences in the chemical composition of 5-HT and NO may contribute to the expression of LTFS over LTS (Susswein et al., 2004). Specifically, the

amount and/or timing of the release of these two neurotransmitters in response to aversive training may facilitate NO dependent LTFS while the effects 5-HT may be nullified by the counteracting effects of FMRFamide (Susswein et al., 2004). Thus, a possible explanation for LTFS in the absence of LTS following massed training may be the continual release of NO versus the counteracting inhibitory effects of FMRFamide on the elevated 5-HT. Future investigation into these mechanisms is required to uncover how these behavioral modifications differ in their energy conservation and in their temporal neurotransmitter availability.

From an evolutionary perspective, it is advantageous for *Aplysia* not to devote limited resources on defensive behaviors such as an enhancement of the TSWR to budget its energy expenditure on more vital behaviors such as feeding (Mac Leod et al., 2018). *Aplysia* evolved in a way that the neurotransmitter responsible for sensitization of defensive responses differs from the neurotransmitters that mediate the more complex feeding neural circuit (Mac Leod et al., 2018). This separation of these two neural circuits greatly benefits *Aplysia* because, following an aversive event, the animal can budget its limited resources to conserve energy that would have been wasted on enhancement of defensive responses to maintain control of feeding behavior (Mac Leod et al., 2018). Thus, another possible explanation for the expression of LTFS in the absence of LTS following massed training may be that the mechanisms that induce LTFS and LTS could also have different temporal circumstances that allow the animal to budget its limited resources towards maintaining feeding behaviors.

The circadian rhythm mediates both LTFS and LTS in *Aplysia*

Objective 1b explored the effects of nocturnal training on LTFS. Previous results indicated that the circadian rhythm strongly modulates LTS in *Aplysia* (Fernandez et al., 2003). However, the gap in knowledge was how does nocturnal training, which does not induce LTS, affect feeding.

Results from **objective 1b** revealed that similar to LTS, the circadian rhythm also modulates LTFS in *Aplysia*. The lack of LTS and LTFS in the nocturnal trained group compared to the diurnal trained group indicate that nocturnal training is not conducive for LTM formation. Unlike LTS and LTFS in the massed vs. spaced training experiment, nocturnal training did not uncouple sensitization from feeding suppression. This discrepancy between the results obtained from **objective 1b** and **objective 1a** suggests that the circadian rhythm has a strong influence on LTM formation by modulating learning in two separate neural circuits. Thus, these findings indicate that nocturnal training is not conducive for LTM formation in both defensive and appetitive behaviors.

These findings that the circadian rhythm modulates two behaviors within *Aplysia* follow general LTM rules that circadian regulation of memory is evolutionarily conserved across many different organisms (Rawashdeh et al., 2018). The hippocampus, which is a complex brain structure in the temporal lobe, is known to have a major role in mammalian learning and memory (Anand and Dhikav, 2012). The hippocampus is essential for consolidating external information into permanent memories stored in the neocortex (Preston and Eichenbaum, 2013). Particularly, in mammals, memory-essential signaling cascades such as cAMP/PKA/PKC and CREB pathways are rhythmically modulated in the hippocampus, which demonstrates the interaction between circadian rhythms and memory across different organisms (Rawashdeh et al., 2018). Thus, the findings from this current study are analogous to the findings across other organisms that demonstrate the role of the circadian rhythm in modulating both behavioral and physiological traces of memory.

Behavioral conclusions

Results from previous experiments as well as results from this **objective 1** have described the many ways in which the amount and pattern of aversive training alter the expression of

defensive and appetitive behaviors. Collectively, these results indicate the adaptability of the animal to respond to many different aversive experiences from their environment. Table 1 below summarizes the expression of two behavioral modifications explored in this study as well as previous work in *Aplysia*. This data provides insight into how the induction and/or persistence of memory depends on the mechanistic differences and the underlying neural circuits modulating behavior. Collectively, all of these previous results coupled with the current results from this study describe how LTM is modulated across many different species, behaviors, and training paradigms (Susswein et al., 2004).

Experimental Protocols	**LTS**	**LTFS**
Spaced training – 16 total trials of training spaced over 4 days	✓	✓
Massed training – 16 total trials of training massed over 1 day	✗	✓
Diurnal training – 4 trials of training administered during day	✓	✓
Nocturnal training – 4 trials of training administered during night	✗	✗
Starvation – 14-day food deprived animals (Mac Leod et al., 2018)	✗	✓

Table 1: Summary of long-term behavioral modification data across different training protocols.

Contributions of transcription and translation to B51 LTDE

Objective 2 investigates whether B51 LTDE is transcription and/or translation dependent by utilizing transcription (ACT-D) and translation (ANI) blockers. Current electrophysiology experiments will be ongoing to fulfill the projected sample size (detailed above). Training-induced TSN LTIE serves as a positive control for the effectiveness of the transcription and translation blockers (Montarolo et al., 1986). Previous research has detailed the molecular pathways for TSN

LTIE and B51 LTDE. 5-HT activates a series of second messengers including cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) to sustain TSN LTIE, which is known to mediate LTS (Kandel et al., 2001; Carew, 2005; Byrne and Hawkins, 2015). The released 5-HT activates adenylyl cyclase which then causes an increase of cAMP (Kandel, 2001; Liu et al., 2008). The increased cAMP triggers the PKA to activate the transcription factor cAMP response element-binding protein (CREB1; Brunelli et al., 1976; Kandel, 2001; Liu et al., 2008). This signaling cascade that mediates LTS is known to require transcription and translation for LTM formation (Montarolo et al., 1986). However, unlike the signaling cascade for TSN LTIE, it was unknown whether the signaling cascade for feeding required similar mechanisms to TSN LTIE. The B51 LTDE signaling cascade is known to be activated by NO, which triggers a series of second messengers including cyclic guanosine monophosphate (cGMP) and protein kinase G (PKG; Antonov et al., 2007; Goldner et al. 2018; Chatterji et al. 2020). Current results from **objective 2** show a trend for B51 LTDE to occur in the T-V group, but not in the T-ACT-D group or the T-ANI group. If statistical significance in this experiment is achieved, this study will indicate the requirements of transcription and translation mechanisms for learning-induced long-term plasticity within the feeding neural circuit of *Aplysia*. Similar to how the TSN LTIE 5-HT-cAMP-PKA pathway requires the activation of transcription factors for LTM, previous research in *Aplysia* has described how the NO-cGMP-PKG activates transcription factors leading to gene expression to sustain long-term-hyper-excitability (LTH) within nociceptive sensory neurons in the pleural ganglia (Lewin and Walters, 1999). This result describes an NO-mediated signaling pathway within the defensive neural circuit that requires gene expression to sustain LTM. Thus, a NO-cGMP-PKG signaling pathway within the feeding neural circuit of *Aplysia* may require the

activation of transcription factors leading to gene expression similar to the NO mediated nociceptive LTH pathway within the defensive neural circuit (Lewin and Walters, 1999).

If the projected statistical significance is not achieved, then this result could show that B51 LTDE may only require a subset of expressed genes or different cellular mechanisms for LTM formation. It is possible that B51 LTDE may only require the translation of pre-existing mRNAs similar to a particular form of LTM in fruit flies (*Drosophila*). The anesthesia-resistant memory (ARM) in *Drosophila* has been shown to be translation dependent for LTM formation, but ARM is not dependent on transcription (Tully et al 1994). Thus, further research is needed to fulfill the projected sample size laid out in this proposed experiment to determine if B51 requires transcription and/or translation. If current trends continue, then we expect B51 LTDE to require both transcription and translation analogous to the defensive neural circuit and TSN LTIE. However, until the projected sample size is met, we cannot rule out the possibility that B51 LTDE may only require a subset of protein synthesis or different cellular mechanisms for LTM formation and retention within the feeding neural circuit.

Long-term memory rules summary and future directions

This project explored whether the feeding neural circuit in *Aplysia* follows general LTM rules by elucidating whether LTFS is induced by massed and nocturnal training (**Objective 1**) and whether its cellular mechanisms require transcription and/or translation (**Objective 2**). Collectively, this project revealed that massed training is another protocol that leads to the expression of feeding suppression in the absence of sensitization, the circadian rhythm modulates both defensive and appetitive behaviors, and that the feeding decision making neuron B51 may require transcription and translation for LTM formation. Thus, these results fill a previous gap in knowledge in how feeding in *Aplysia* is mediated by general LTM rules.

Collectively, this project explored three LTM rules within the reductionistic memory model organism: *Aplysia californica*. We explored how feeding is very diverse and complex for LTM formation. This study showed how feeding follows some general LTM, but not all of them. Future directions from this study will uncover how feeding is mediated across many other higher organisms. For instance, previous research in mice has shown that the hippocampus plays a vital role in LTM formation for defensive behaviors (Malleret et al., 2001; Kandel, 2001). Similarly, this previous study revealed the importance of PKA for LTM formation, which is analogous to memory storage in *Aplysia* and *Drosophila* (Kandel, 2001). These findings demonstrate how universal LTM rules can be explored across many different reductionistic memory model organisms such as mice, *Drosophila,* and *Aplysia*. However, feeding LTM rules have yet to be fully explored throughout these systems. Thus, future directions from this study will reveal further mechanisms for long-term memory formation and retention both in *Aplysia* and the universality of memory across many different organisms.

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