THE ROLE OF NITRIC OXIDE SIGNALING IN LEARNING-INDUCED BEHAVIORAL PLASTICITY AND NEURAL CIRCUIT DYNAMICS IN THE SEA HARE APLYSIA CALIFORNICA

A Thesis

by

JESSE FARRUGGELLA

Submitted to the Office of Graduate and Professional Studies of Texas A&M University
and the Graduate Faculty of The Texas A&M University – Corpus Christi
in partial fulfillment of the requirements for the joint degree of

MASTER OF SCIENCE

Chair of Committee,	Riccardo Mozzachiodi
Committee Members,	Kirk Cammarata
	Manuela Gardner
Head of Department,	Joe Fox

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Major Subject: Marine Biology

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Approved as to style and content by:

Dr. Riccardo Mozzachiodi
(Chair of Committee)

Dr. Kirk Cammarata
(Member)

Dr. Manuela Gardner
(Member)

Dr. Joel Fox
(Department Chair)

Dr. Frank Pezold
(Dean, College of Science & Engineering)

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ABSTRACT

THE ROLE OF NITRIC OXIDE SIGNALING IN LEARNING-INDUCED BEHAVIORAL PLASTICITY AND NEURAL CIRCUIT DYNAMICS IN THE SEA HARE *APLYSIA CALIFORNICA*

Jesse Farruggella, B.S. Marine Biology, B.A. Chemistry with Environmental Concentration, Roger Williams University

Chair of Advisory Committee: Dr. Riccardo Mozzachiodi

Animals constantly regulate their behaviors in response to environmental stimuli. These adjustments involve an active and dynamic balance between defensive and non-defensive behaviors, and underlying each change are the fundamental alterations within the neural circuits that form the cellular bases for those behaviors. The marine mollusk *Aplysia californica* provides an ideal model system for examining plasticity at both the behavioral and neural levels following exposure to aversive stimuli. After receiving a noxious stimulus, *Aplysia* elicit the learned behavioral change known as sensitization through which their defensive responses are enhanced, in particular the tail-siphon withdrawal reflex (TSWR). Sensitization of the TSWR manifests from an increase in facilitation at the neuronal synapses that govern the reflex. Sensitization training induces a concurrent suppression of non-defensive behaviors, particularly feeding behavior. The suppression of feeding results from a decrease in excitability of neuron B51, which is a key decision-making neuron in the feeding neural circuit that is critical to the production of feeding behavior. The neurotransmitter serotonin has been shown to mediate and induce the sensitization of the TSWR, but it is not involved in the concomitant suppression of feeding behavior. This study proposed the neurotransmitter

nitric oxide (NO) as the signaling molecule that mediates the modification of feeding. The goal of this study was to examine the roles of NO signaling in the enhancement of the TSWR produced by sensitization training, the sensitization training-induced suppression of feeding, and the modulation of the feeding neural circuit. NO signaling was found to be necessary for sensitization training-induced behavioral plasticity. When NO signaling was pharmacologically blocked by *in vivo* application of the NO synthase inhibitor N_{ω}-nitro-L-arginine methyl ester (L-NAME), sensitization training failed to produce the sensitization of the TSWR. L-NAME also prevented sensitization training from inducing the suppression of feeding behavior known to occur concomitantly with the sensitization of the TSWR. These results suggested that modifications of both defensive and non-defensive behaviors via aversive learning were prevented when NO signaling was blocked. NO signaling was also found to modulate the *Aplysia* feeding neural circuit. NO is known to tonically inhibit feeding behavior *in vivo*. The application of L-NAME *in vitro* to isolated preparations of the feeding circuit caused the excitability and activity of neuron B51 to increase. Conversely, when NO signaling was augmented by *in vitro* application of the NO donor *S*-nitroso-*N*-acetyl-penicillamine (SNAP) to the isolated feeding circuit, the excitability of B51 decreased in a manner analogous to that produced by sensitization training. Neither L-NAME nor SNAP treatments affected the resting membrane properties of B51, indicating that the bidirectional modulation of B51 excitability by NO signaling acts through voltage-dependent channels.

Based on these results, it is evident that NO signaling modulates the changes in multiple behaviors following exposure to aversive stimuli, and that NO signaling is significantly involved in and essential for behavioral and cellular plasticity in *Aplysia*.

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INTRODUCTION

In order to survive, an organism must be able to respond to its environment. These responses are exemplified by predator-prey interactions, in which prey organisms adapt defenses through behavioral, morphological, and/or physiological alterations – a phenomenon known as phenotypic plasticity (Miner et al., 2005; Brookes and Rochette, 2007). The changes involve an innate risk assessment and a decision to optimize survival by enhancing defensive actions, such as increasing vigilance, at the cost of other, non-defensive functions, such as feeding and reproduction (Milinski, 1985; Lima and Bednekoff, 1999; Kavaliers and Choleris, 2001). These behavioral modifications can be influenced by an organism's experiences (Turner et al., 2006), and augmenting defensive responses when facing a threat is recognized to increase survivability (Blanchard et al., 2011; Crook et al., 2014). To date, still little is known regarding how an animal decides to regulate defensive and non-defensive behaviors simultaneously as well as the underlying physiological changes that occur within the animal's behavioral neural circuits that culminate in this regulation. However, it is known that plasticity in behavior is a consequence of the plasticity in neurons and synapses that is fundamental to learning and memory.

The benefits of using Aplysia

The investigations of the cellular and molecular bases of learning and memory formation involve the use of animal models, vertebrates and invertebrates alike (Castellucci, 2008). The marine mollusk *Aplysia californica* (hereafter *Aplysia*) has proven ideal for the study of the neurophysiological substrates of learning and memory

through numerous, significant advantages. Neurons have properties that are relatively conserved across both vertebrates and invertebrates, such as basic signaling, neurophysiological functions, and learning mechanisms (Kandel, 2001). *Aplysia* only has approximately 20,000 neurons, which in general are comparably large, readily identifiable, and clustered into ganglia (Fig. 1), as opposed to the billions of neurons within the highly complex vertebrate nervous systems (Kandel, 2001; Hawkins et al., 2006). Also, *Aplysia* exhibit defensive and non-defensive behaviors – particularly withdrawal reflexes and feeding, respectively – that are easy to evoke and evaluate (Kupfermann, 1974). In addition, the neural circuits underlying these behaviors are well-characterized (Cleary et al., 1998; Cropper et al., 2004a), enabling the extensive investigation of the cellular and molecular mechanisms that modulate behaviors in *Aplysia*.

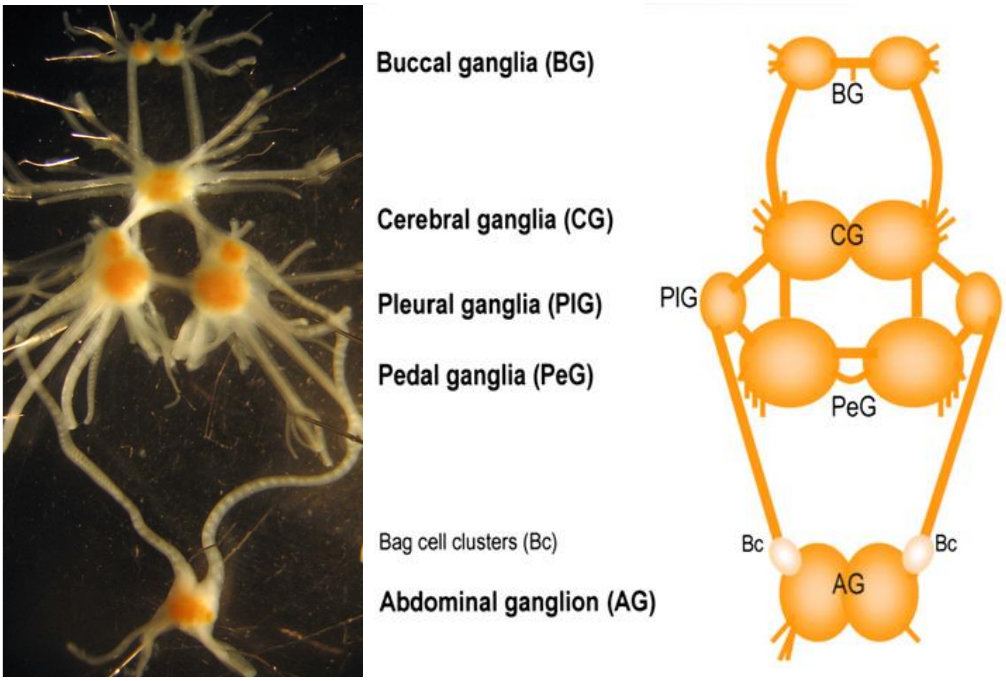

Figure 1. The *Aplysia* nervous system. The left image is © Jian Jing 2012; the right image is modified from Moroz et al. (2006).

Through their defensive reflexes and feeding behaviors, *Aplysia* have demonstrated their capabilities for both associative and nonassociative learning, a repertoire also present in vertebrates, including humans (Pinsker et al., 1970; Colwill et al., 1988b). In general, associative learning includes classical conditioning, in which a predictive relationship is established between two stimuli/events, and operant conditioning, in which a predictive relationship is established between a behavior and a reinforcing consequence (Thorndike, 1911; Pavlov and Anrep, 1927; Skinner, 1938). Conversely, nonassociative learning includes habituation, which is a progressive decline in response to repeated stimuli, and sensitization, a learned fear in which a response to a previously weak or neutral stimulus is augmented by a strong or noxious stimulus (Hilgard and Marquis, 1961; Thompson and Spencer, 1966; Groves and Thompson, 1970).

In the *Aplysia* model, defensive behaviors exhibit classical and operant conditioning (Carew et al., 1981; Colwill et al., 1988a; Antonov et al., 2001; Kandel, 2001; Hawkins et al., 2006), as well as habituation and sensitization (Pinsker et al., 1970; Pinsker et al., 1973; Byrne et al., 1978; Bailey and Chen, 1983; Hawkins et al., 1998; Kandel, 2001). *Aplysia* feeding behaviors can also undergo classical and operant conditioning (Lechner et al., 2000a; Brembs et al., 2002; Baxter and Byrne, 2006; Lorenzetti et al., 2006; Mozzachiodi et al., 2008). Additionally, these associative and nonassociative learning processes can produce short- and long-term forms of memories (Botzer et al., 1998; Lechner et al., 2000a; Brembs et al., 2002; Katzoff et al., 2002; Hawkins et al., 2006) which reflect the basal short- and long-term forms of neuronal and synaptic plasticity (Casadio et al., 1999; Katzoff et al., 2006). Further, the learned

modifications that occur at the behavioral level *in vivo* are also seen *in vitro* in reduced, dissociated preparations of *Aplysia* circuits, whether the circuit is taken from an animal that has undergone a learning protocol, i.e., correlate (Lechner et al., 2000b; Brembs et al., 2002; Lorenzetti et al., 2006), or the circuit itself was subjected to an *in vitro* learning protocol without the animal, i.e., analog (Nargeot et al., 1997; Mozzachiodi et al., 2003; Lorenzetti et al., 2006; Mozzachiodi et al., 2008).

Defensive behavior: Tail-siphon withdrawal reflex

Sensitization has been extensively studied in *Aplysia* using the defensive behaviors of the animal. Sensitization induces a heightened state of arousal that can be characterized by the expression of defensive responses (Walters et al., 1981). The defensive withdrawal responses of *Aplysia* have been paramount in studying sensitization (Walters et al., 1983b; Cleary et al., 1998; Casadio et al., 1999; Kandel, 2001; Hawkins et al., 2006). When *Aplysia* receive a noxious stimulus to the tail or body wall – such as an electric shock mimicking a predator attack (Watkins et al., 2010) – they elicit a coordinated, defensive response that involves the transient withdrawal of the tail, siphon, and gill. A particular constituent of this response that is of distinguished neurophysiological significance is the tail-siphon withdrawal reflex (TSWR), in which stimulation of the tail leads to the withdrawal of the siphon (Goldsmith and Byrne, 1993; Hawkins et al., 2006).

The TSWR is enhanced through sensitization, and the change in duration of the withdrawal reflex serves as a measure of the sensitization (Scholz and Byrne, 1987). The duration of the sensitization memory depends on the stimulation protocol (Botzer et al.,

1998; Kandel, 2001), and it ranges from single-stimulus-induced sensitization lasting minutes to hours (Pinsker et al., 1970), to multiple-stimuli-induced long-term sensitization (LTS) lasting up to several days (Pinsker et al., 1973; Cleary et al., 1998; Wainwright et al., 2002; Khabour et al., 2004; Acheampong et al., 2012; Shields-Johnson et al., 2013). Furthermore, the TSWR is an ipsilateralized response mediated by tail sensory neurons in the left and right pleural ganglia that synapse – directly through monosynaptic connections, and indirectly through heterosynaptic connections via modulatory interneurons (Fig. 2) – with tail motor neurons in the left and right pedal and abdominal ganglia (Walters et al., 1983a, b; Scholz and Byrne, 1987; Goldsmith and Byrne, 1993; Cleary et al., 1998; Kandel, 2001). Sensitization is associated with the augmentation of these synaptic connections and the increase in excitability of the tail sensory neurons (Montarolo et al., 1986; Scholz and Byrne, 1987; Bailey and Chen, 1988; Cleary et al., 1998; Marinesco and Carew, 2002). The enhancements of synaptic transmission and neuron excitability are accomplished within the TSWR neural circuit through the release and subsequent binding of specific neurotransmitters triggered by noxious stimuli.

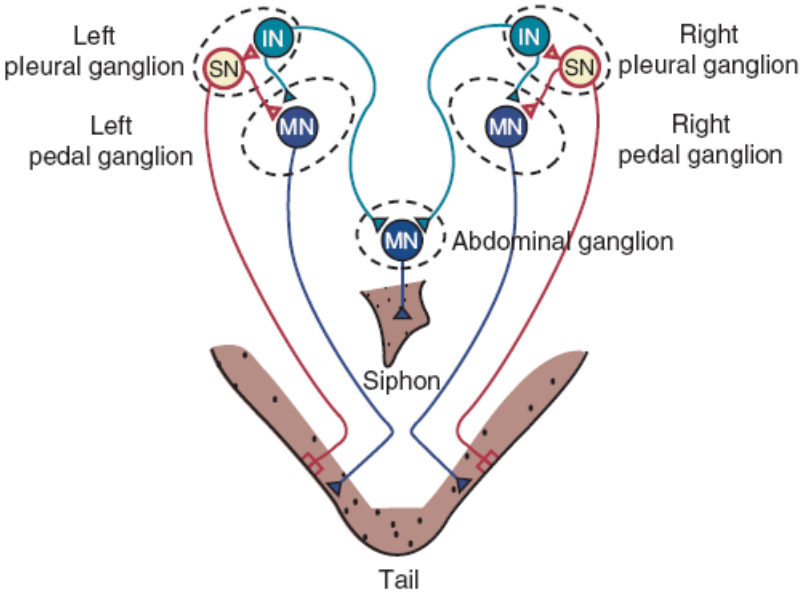

Figure 2. The neural circuit underlying the TSWR. Tail stimulation activates sensory neurons (SN) in the pleural ganglion. These SN activate a polysynaptic pathway via interneurons (IN) that projects to motor neurons (MN) in the abdominal ganglion, which in turn elicit the withdrawal of the siphon. The colored lines indicate the respective synaptic pathways. Image modified from Byrne et al. (2008).

Non-defensive behavior: Feeding

The non-defensive behaviors of *Aplysia* and their underlying neural circuits are also significant for investigating the neurophysiological changes that result in behavioral modifications (Baxter and Byrne, 2006). One particular and well-characterized non-defensive behavior of interest is feeding. The initial, appetitive phase of feeding is a positive chemotactic response that consists of locomotion to bring the animal near its food source (seaweed) followed by head waving to orient the animal to its food. Head waving is a distinctive posture in which the *Aplysia* lifts the anterior two-thirds of its body and moves it in arcing motions (Kupfermann, 1974). Contact with food leads to the consummatory phase of feeding that involves ingestion of the food and typically

maintains the outstretched posture. The ingestive biting behavior is highly stereotyped; ingestion begins with the protraction of the odontophore and radula (Fig. 3A), followed by the opening and subsequent closure of the radula around food (Fig. 3B), and ends with the retraction of the odontophore and radula (Fig. 3C) bringing the food back into the buccal cavity (Kupfermann, 1974). These consummatory bites occur in an all-or-nothing rhythmic process governed by a central pattern generator (CPG) within the neuronal circuits of the buccal ganglion (Fig. 4) (Kupfermann, 1974; Nargeot et al., 1997; Lechner et al., 2000a; Brembs et al., 2002; Cropper et al., 2004b). Egestive behavior occurs should the *Aplysia* consume or begin to consume an object deemed inedible by the esophageal nerves (Schwarz and Susswein, 1984, 1986). The rejection response mirrors the process of ingestive behavior (i.e., biting), save for the reversal of radula opening and closure (Kupfermann, 1974; Brembs et al., 2004). Notably, the neural mechanisms of these patterned ingestive and egestive actions continue to be expressed *in vitro* by dissociated buccal ganglia, producing ingestive buccal motor patterns (iBMPs) and egestive buccal motor patterns (eBMPs) that represent fictive feeding and rejection actions correlating to *in vivo* feeding and rejection movements, respectively (Morton and Chiel, 1993a, b; Nargeot et al., 1997, 1999a, b). Analogous to the TSWR and sensitization, feeding behavior in *Aplysia* exhibits protocol-dependent short- and long-term memory (Lechner et al., 2000a; Brembs et al., 2002; Katzoff et al., 2002; Acheampong et al., 2012).

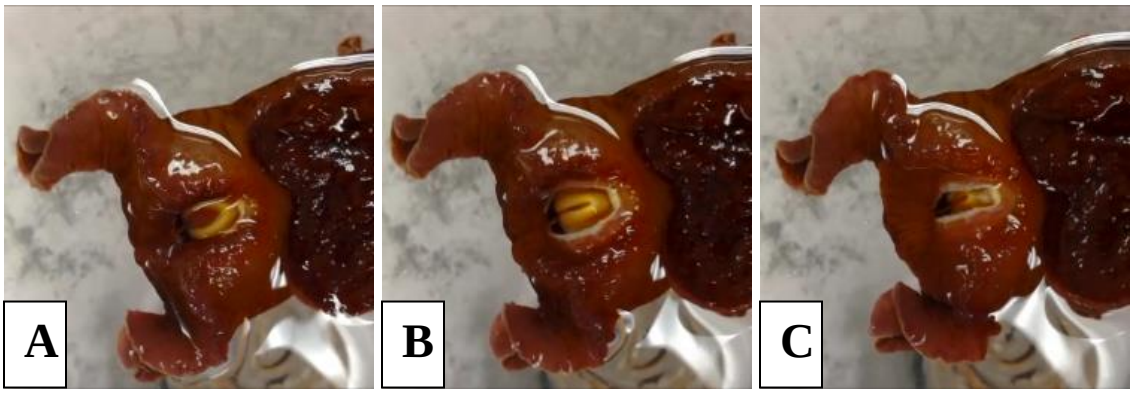

Figure 3. The full pattern of radula movements involved in *Aplysia* ingestive biting behavior: radula protraction (A), closure (B), and retraction (C).

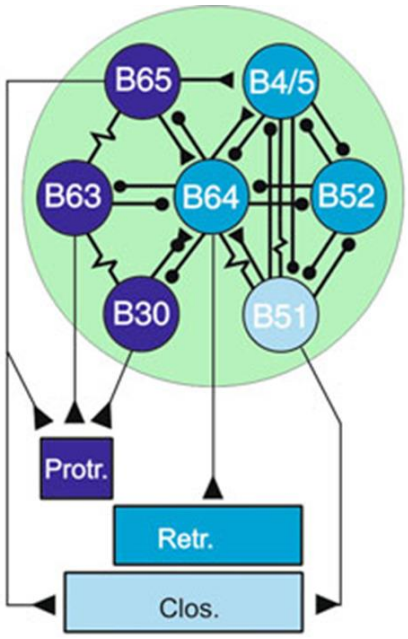

Figure 4. The neural circuit underlying the feeding CPG. The CPG includes subunits controlling radula protraction (Protr.; dark blue) and retraction (Retr.; blue), in addition to neuron B51 that controls radula closure (Clos.; light blue). The lines denote synaptic pathways and control of respective radula movements (Nargeot and Simmers, 2011).

One critical component of the feeding CPG is the decision-making cell buccal neuron 51 (B51) (Nargeot et al., 1999a, b; Baxter and Byrne, 2006; Lorenzetti et al., 2006; Mozzachiodi et al., 2008). The activity of B51 dictates the type of BMP elicited and therefore the type of feeding behavior evoked, with depolarization leading to iBMPs

and hyperpolarization inhibiting iBMPs (Nargeot et al., 1999b). When triggering iBMPs, B51 exhibits a characteristic, sustained, all-or-nothing response referred to as a plateau potential (Fig. 5) (Plummer and Kirk, 1990; Nargeot et al., 1999a, b). B51 activity is ultimately dependent upon its intrinsic membrane properties, such input resistance (R_{in}; i.e., a measure of the number of open channels at rest) and burst threshold (BT; i.e., the amount of depolarizing current necessary to elicit the distinctive plateau potential), which can be modified by learning, making B51 a locus of plasticity for changes in feeding behavior (Nargeot et al., 1999a, b; Baxter and Byrne, 2006; Mozzachiodi et al., 2008). For example, B51 excitability and activity increase when R_{in} increases and BT decreases, which in turn yields more iBMPs and elicits more bites by the animal (Nargeot et al., 1999a, b; Kabotyanski et al., 2000; Brembs et al., 2002). Conversely, B51 excitability and activity decrease when BT increases, producing fewer iBMPs and therefore fewer bites (Baxter and Byrne, 2006; Shields-Johnson et al., 2013). Thus, B51 is a prominent and neurophysiologically significant site for examining feeding behavior in *Aplysia*.

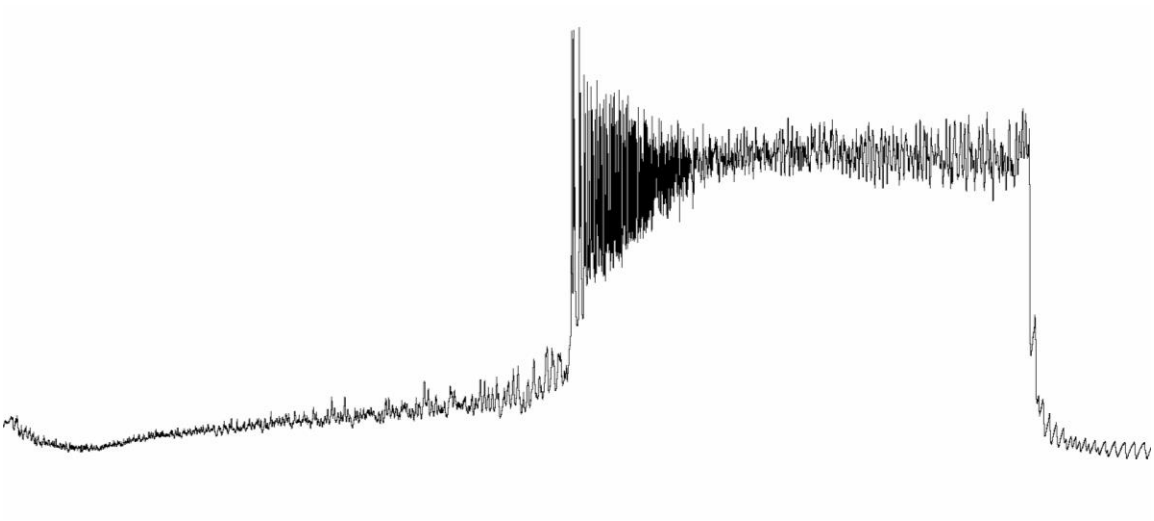

Figure 5. A representative intracellular recording from B51 illustrating the characteristic plateau potential.

The interaction of defensive and non-defensive behaviors

While much is known about learning-induced synaptic changes in the TSWR and feeding neural circuits when they are considered independently (Cleary et al., 1998; Baxter and Byrne, 2006; Mozzachiodi et al., 2013), the mechanisms of behavioral and neural plasticity arising from stimuli modulating multiple responses from these separate yet interconnected circuits have only begun to be addressed. The interaction of these defensive and non-defensive behaviors becomes evident when *Aplysia* are exposed to a noxious stimulus and consequently enhance their defensive reflexes. The cost incurred to the *Aplysia* by enhancing a defensive response, such as the TSWR, through sensitization is the concomitant suppression of non-defensive behaviors, particularly feeding (Acheampong et al., 2012). After exposure to noxious stimuli that produce sensitization, the BT of B51 significantly increases, corresponding to decreased excitability and potentially fewer plateau potentials, fewer iBMPs, and fewer bites (Shields-Johnson et al., 2013), leading to an induced suppression of feeding behavior (Acheampong et al., 2012). In *Aplysia* subjected to a LTS training protocol, these changes are visible for at least 24 h, and *Aplysia* that undergo single-trial training produce the aforementioned changes visible for at least 2 h (Cleary et al., 1998; Acheampong et al., 2012; Shields-Johnson et al., 2013).

The neurotransmitter serotonin (5-HT) is involved in mediating sensitization of the TSWR (Glanzman et al., 1989; Levenson et al., 1999; Kandel, 2001). When an *Aplysia* receives sensitizing stimuli on its tail or body wall, 5-HT is released into the hemolymph and neuropil in an area localized around the pleural and pedal ganglia on the side ipsilateral to the stimulus (Levenson et al., 1999; Marinesco and Carew, 2002).

Further, the magnitude of elevation in humoral 5-HT concentration directly correlates to the increase in duration of siphon withdrawal and consequently the degree of sensitization (Levenson et al., 1999). The increase in 5-HT induces synaptic facilitation between the tail sensory and tail motor neurons (Mackey et al., 1989). Notably, exogenous 5-HT applied to tail sensory neurons and sensorimotor synapses, whether by injection *in vivo* or administration *in vitro*, simulates the facilitation produced by sensitizing stimuli (Brunelli et al., 1976; Walters et al., 1983b; Montarolo et al., 1986; Glanzman et al., 1989; Casadio et al., 1999; Marinesco and Carew, 2002). The neurotransmitter 5-HT additionally appears to modulate the latency for feeding in *Aplysia* (Levenson et al., 1999), but the application of exogenous 5-HT, which is sufficient to induce sensitization 24 h after treatment (i.e., LTS; Levenson et al., 2000), failed to replicate the suppression of feeding behavior that normally occurs concurrently with sensitization (Shields-Johnson et al., 2013). The application of 5-HT also failed to distinctly alter BT and decrease the excitability of B51 (Shields-Johnson et al., 2013). Clearly, 5-HT accounts for only a portion of the interaction between the TSWR and feeding behavior.

Upon initial review, the neurotransmitter dopamine (DA) appears to be a possible candidate for this bridging signaling molecule. DA functions in reward pathways under associative learning in feeding behavior, serving as a reinforcement neurotransmitter following successful ingestion (Baxter and Byrne, 2006; Susswein and Chiel, 2012). DA is also a key signaling molecule involved in modulating the feeding CPG (Nargeot et al., 1999c). The application of DA *in vitro* to the buccal ganglion activated the CPG, initiating the patterned buccal motor output with a bias toward iBMPs (Kabotyanski et

al., 2000). However, during the DA-induced rhythmic activity, B51 experienced reduced excitability and was inactive (Kabotyanski et al., 2000); thus DA signaling does not involve the site responsible for plasticity underlying learning and modification of feeding behavior (Nargeot et al., 1999a, b; Baxter and Byrne, 2006; Mozzachiodi et al., 2008). Further, DA presynaptically inhibits the release of neurotransmitters from siphon sensory neurons (Glanzman et al., 1989), precluding the synaptic facilitation that is integral to learning and modification of the TSWR (Scholz and Byrne, 1987; Bailey and Chen, 1988; Cleary et al., 1998).

Therefore, there likely exists another signaling molecule that couples sensitization and feeding suppression in *Aplysia*. To accomplish such a link, the neurotransmitter would need to be involved in the response to aversive stimuli as well as the modulation of feeding behavior through learning. Nitric oxide, a known signaling molecule that possesses these characteristics, may function as that bridge.

Bridging the behavioral alterations: Nitric oxide

The gaseous free radical nitric oxide (NO) is an unconventional yet nearly ubiquitous neurotransmitter (Jacklet, 1997). NO is a small and transient molecule, rendering it unfeasible to study by actively tracking its production and concentration *in situ*; therefore, the study of NO is typically accomplished by pharmacologically inhibiting or augmenting NO signaling (Hou et al., 1999; Cristino et al., 2008). NO is produced from arginine by the enzyme nitric oxide synthase and serves a wide variety of functions across different systems (Fig. 6) (Garthwaite and Boulton, 1995; Koh and Jacklet, 1999). In mammals, NO acts as a mediator of vasodilation and smooth muscle relaxation, is

utilized in the immune system as a cytotoxic defense, and operates as a neurotransmitter in the visual system (Hou et al., 1999; Jacklet and Koh, 2001; Cristino et al., 2008). In invertebrates, NO also participates in conveying sensory information (Jacklet, 1997). More significantly, however, NO functions in neural pathways that modulate the release and reception of neurotransmitters, culminating in synaptic plasticity that underlies learning and memory (Jacklet, 1997). NO performs a crucial role in long-term potentiation in the mammalian hippocampus mediated through the NO-cyclic guanosine monophosphate (cGMP)-cGMP dependent protein kinase (PKG) pathway (Zhuo et al., 1994; Hopper and Garthwaite, 2006). In the marine mollusk *Octopus vulgaris*, NO is essential for tactile and visual learning and memory (Robertson et al., 1994; Robertson et al., 1996).

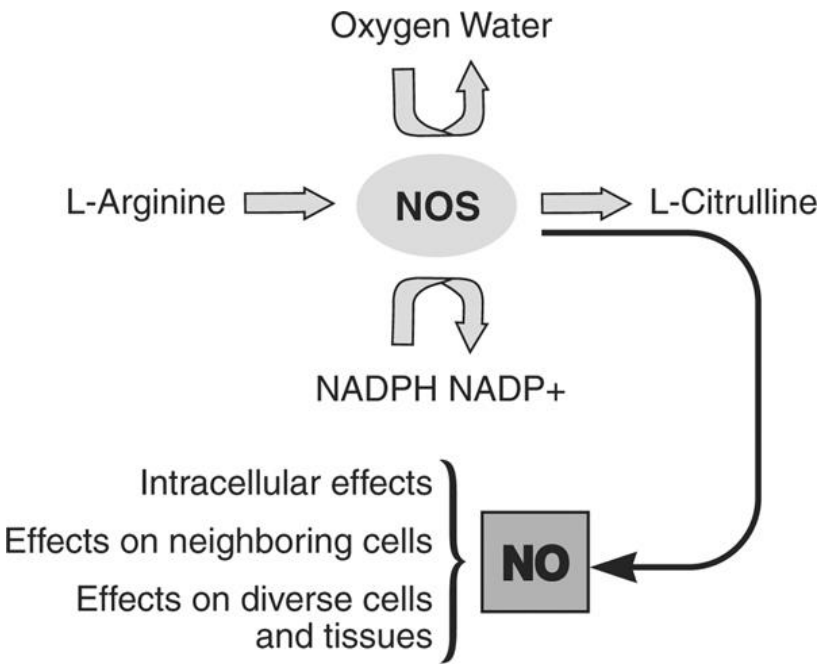

Figure 6. The production of nitric oxide (NO). Abbreviations: NOS, nitric oxide synthase; NADPH, nicotinamide adenine dinucleotide phosphate (Mackenzie et al., 2008).

In *Aplysia*, NO is involved in the neuronal circuits that govern feeding behavior (Katzoff et al., 2002). In the feeding neural circuit, NO is produced and maintained at basal, tonic levels at which it inhibits both appetitive and consummatory feeding behaviors in the absence of food stimuli (Miller et al., 2011a; Miller et al., 2011b). However, in the excitatory presence of food stimuli, this background inhibition is overridden (Miller et al., 2011a; Miller et al., 2011b). Many neurons associated with governing feeding behavior in *Aplysia* have been linked to NO, such as the nitrergic cerebral ganglion neuron C2 that excites the metacerebral cell (MCC), which is involved in the arousal to food stimuli that overcomes the tonic inhibition of NO (Katzoff et al., 2006; Miller et al., 2011b). NO is produced by several neurons in the cerebral and buccal ganglia that control key aspects of feeding behavior, such as the cerebral-pedal regulator neuron (C-PR), a command neuron that also excites the MCC during food arousal, and the coupled buccal neurons B31/32, which are part of the feeding CPG and are involved in generating iBMPs (Moroz, 2006; Hurwitz et al., 2008; Ye et al., 2009; Miller et al., 2011a). Additionally, NO synthesis and transmission are essential for *Aplysia* to learn that an object or food item is inedible and to form short- and long-term associative memory relating to this feeding behavior (Katzoff et al., 2002; Katzoff et al., 2006). Further, NO is part of a pathway engaged in enhancing synaptic transmission and increasing excitability of *Aplysia* nociceptive sensory neurons that functions similarly to the pathway managing serotonin-mediated sensitization (Lewin and Walters, 1999). Finally, NO is involved in mediating synaptic facilitation of the sensory neurons controlling the siphon withdrawal reflex through classical conditioning (Antonov et al., 2007).

This study sought to continue to investigate the relationship between two plastic behaviors: the TSWR and feeding. In particular, this study aimed to address and account for the suppression of consummatory feeding behavior that occurs concomitantly following exposure to sensitization-inducing stimuli in *Aplysia* with a focus on the role of NO. This study also investigated the involvement of NO signaling in the LTS of the TSWR, as well as the role of NO signaling in the feeding neural circuit.

Objectives

Objective 1: Explore the function of NO in the behavioral changes produced by LTS training. In this objective, the role of NO in mediating the effects of LTS training on the TSWR (Objective 1.1) and feeding (Objective 1.2) was analyzed. The NO signaling pathway was blocked through the inhibition of NO synthase via injection of N_{ω}-nitro-L-arginine methyl ester (L-NAME) solution. Control animals were injected with a vehicle medium that was an isotonic, artificial seawater (ASW) solution containing a normal concentration of cations (Normal ASW; comprised of 450 mM NaCl, 10 mM KCl, 30 mM $MgCl_2$, 20 mM $MgSO_4$, 10 mM $CaCl_2$, and 10 mM HEPES, with pH adjusted to 7.5 using NaOH). Four experimental groups were used in this objective: L-NAME-injected trained, L-NAME-injected untrained, Normal ASW-injected trained, and Normal ASW-injected untrained *Aplysia*. These experimental groups are hereafter denoted as L-NAME-trained, L-NAME-untrained, vehicle-trained, and vehicle-untrained, respectively.

Objective 1.1: Examine the role of NO in the formation of LTS of the TSWR produced by LTS training.

Objective 1.2: Analyze the role of NO in the feeding suppression produced by LTS training.

Objective 2: Explore the function of NO in the behavioral changes produced by single-trial sensitization training. In this objective, the role of NO in mediating the effects of single-trial training on feeding was analyzed. Single-trial training is known to induce behavioral changes observable at 15 min and 2 h post-training. The NO signaling pathway was blocked through the inhibition of NO synthase via injection of L-NAME. Control animals were injected with a vehicle solution (Normal ASW). Four experimental groups were used in this objective: L-NAME-trained, L-NAME-untrained, vehicle-trained, and vehicle-untrained.

Objective 3: Characterize the role of NO in modulating B51 excitability and activity. The role of NO in regulating feeding behavior was analyzed *in vitro* by investigating the differences in B51 excitability and activity in naïve B51 neurons that had not undergone any behavioral testing or sensitization training protocols.

Objective 3.1: Investigate the role of NO in the alteration of B51 excitability and activity in naïve *Aplysia* neurons by blocking the NO signaling pathway through the inhibition of NO synthase via *in vitro* bolus application of L-NAME (or vehicle for controls).

Objective 3.2: As an addendum to the primary focus of the effects of blocking the NO signaling pathway via L-NAME (Objective 3.1), the NO signaling pathway was augmented through the addition of exogenous NO via *in vitro* bolus application of the NO donor molecule *S*-nitroso-*N*-acetyl-penicillamine (SNAP; or vehicle for controls).

MATERIALS AND METHODS

Logistics

All experiments were performed in the Mozzachiodi research laboratory at Texas A&M University Corpus Christi. All experimental animals (*Aplysia californica*) were kept in a tank laboratory adjacent to the research lab. The laboratory was properly equipped for the proposed experiments, and all experiments were approved by the Institutional Animal Care and Use Committee (IACUC #28-13).

Adult *Aplysia californica* with masses between 100-200g were obtained from Marinus Scientific (Garden Grove, CA) and South Coast BioMarine LLC (San Pedro, CA). The *Aplysia* were housed within two 530L aquaria that continuously circulated, filtered, and aerated artificial seawater (ASW; Instant Ocean, Aquarium Systems, Mentor, OH) maintained at 15°C and 34-36ppt salinity to match the *Aplysia* habitat. Upon arrival, the *Aplysia* were removed from their shipping container and equilibrated to the tank temperature for 30 min while remaining in their original, individual, sealed plastic bags (Levenson et al., 1999). After equilibration, the *Aplysia* were removed from their bags, gently cleaned of debris, and transferred to individual, custom-perforated, plastic cages each labelled with the shipment arrival date and a unique letter. The animals were maintained on a 12-h/12-h light/dark cycle (Kupfermann, 1974). The animals were allowed to acclimate to tank conditions for at least 3 days before any use in or preparation for use in experiments (Levenson et al., 1999), and since this species of *Aplysia* is diurnally active, all experiments occurred during daylight hours (Kupfermann, 1974). Each animal was fed a strip of dried seaweed (0.7g, 3.5cm x 19.3cm; Emerald

Cove Organic Pacific Nori, Great Eastern Sun, Asheville, NC) 3 times per week (Monday, Wednesday, Saturday) in absence of an experiment-specific feeding schedule.

Tank water properties (nitrate, nitrite, ammonia, and phosphate levels) were routinely checked via Aquarium Pharmaceuticals (API) saltwater aquaria testing kits (Mars Fishcare, Inc., Franklin, TN) and were kept at optimal levels for animal health using water changes with prepared ASW (34-36ppt salinity), regular tank cleaning (i.e., removal of animal waste), and AmQuel Plus water conditioner (Kordon LLC, Hayward, CA). The pH of the tanks was routinely checked via S20 SevenEasy pH meter (Mettler Toledo, LLC, Columbus, OH) and maintained near 8.3 (the pH of the *Aplysia* habitat) using Pro Buffer dKH (Kent Marine, Central Garden & Pet Company, Walnut Creek, CA) and water changes with prepared ASW. Salinity was also routinely checked via Atago ATC-S/Mill-E handheld refractometer (ATAGO U.S.A., Inc., Bellevue, WA) and maintained at 34-36ppt using water changes with prepared ASW.

Each animal was deprived of food for 48 h immediately prior to its use in an experiment and throughout the duration of the experiment to ensure that all experimental animals were in a similar motivational state (Kupfermann, 1974; Nargeot et al., 1997; Lechner et al., 2000a) and to prevent food ingestion/satiation from influencing the animal's responses to behavioral testing stimuli (Advokat, 1980). Egg-laying also inhibits defensive and feeding behaviors, potentially suppressing LTS and its effects (Goldsmith and Byrne, 1993). Therefore, any *Aplysia* that laid eggs was not used in an experiment until 7 days had passed to ensure that the humoral levels of bag cell peptides – which, when elevated, elicit the behavioral modifications preceding and accompanying egg-laying in *Aplysia* – had returned to baseline concentration (Goldsmith and Byrne,

1993). Further, if an animal released ink or opaline at any point during the procedures performed to prepare an animal for behavioral testing or during the pre-test portion of behavioral testing, that animal was removed from the experiment due to the indication of prior sensitization or impaired health (Scholz and Byrne, 1987; Wainwright et al., 2002).

Experimental Design

Preparation of animals used in LTS training experiments (Objective 1)

Seaweed extract feeding test: Seaweed extract (SWE) served the purpose of reliably eliciting consummatory feeding behavior in the absence of tangible food to prevent food ingestion/satiation from influencing the animal's responses to behavioral testing stimuli (Advokat, 1980). SWE was prepared fresh daily by soaking and stirring a half-sheet of dried seaweed (10.5cm x 19.3cm; Emerald Cove Organic Pacific Nori) in 300mL of ASW for 30 min and then filtering the contents through a standard coffee filter. For the SWE feeding test, an animal was placed into a glass pedestal bowl (diameter 17.8cm) containing 167mL of SWE and 1333mL of ASW for 1500mL of SWE solution (Fig. 7) with a concentration of 1 part SWE to 8 parts ASW (Brembs et al., 2002; Acheampong et al., 2012). The 5 min duration for the feeding test began when the animal was fully attached to the glass bowl, and the total number of bites elicited was recorded (Kupfermann, 1974; Brembs et al., 2002). For a bite to be counted, it must have exhibited the full pattern of radula movements (Fig. 3) involved in ingestive biting behavior (Kupfermann, 1974).

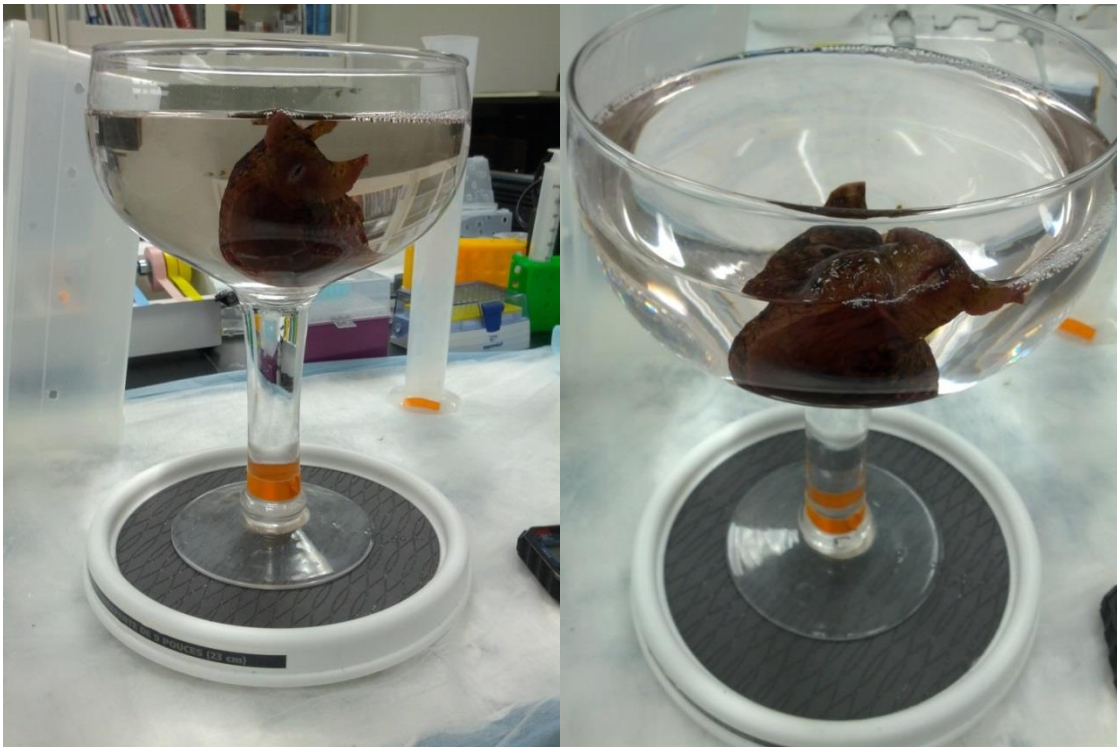

Figure 7. An *Aplysia* exposed to SWE in the feeding test. The glass pedestal bowls enable the observation of the *Aplysia* feeding behavior.

Parapodectomy: Aplysia underwent a surgical procedure to enhance visualization of the siphon (Fig. 8) (Cleary et al., 1998; Wainwright et al., 2002). Prior to the parapodectomy, a SWE feeding test was first performed to assess the relative health of the animal; if the animal generated at least 10 bites during the 5 min, it was considered to have "passed" (Acheampong et al., 2012; Shields-Johnson et al., 2013). Animals were temporarily anaesthetized by burying them under ice for 18 min (Goldsmith and Byrne, 1993; Acheampong et al., 2012). Ice provided an anesthetic method that was much less invasive and more rapidly reversed (i.e., removing the animal from the ice to return to normal temperature) than pharmacological means. The surgery proceeded only if the animal did not respond to tactile stimuli, e.g., lightly brushing a rhinophore (Lechner et

al., 2000a), otherwise it was placed back under ice for 3 to 5 min longer. The parapodia, paired extensions of the body wall surrounding the mantle cavity, were deflected away from the mantle opening. The posterior portion of each parapodium was clamped with a hemostat just above where it met the body wall and then surgically removed. The clamping allowed the parapodia to be cut without the subsequent loss of hemolymph. The animals were given at least 7 days to recover from the parapodectomy.

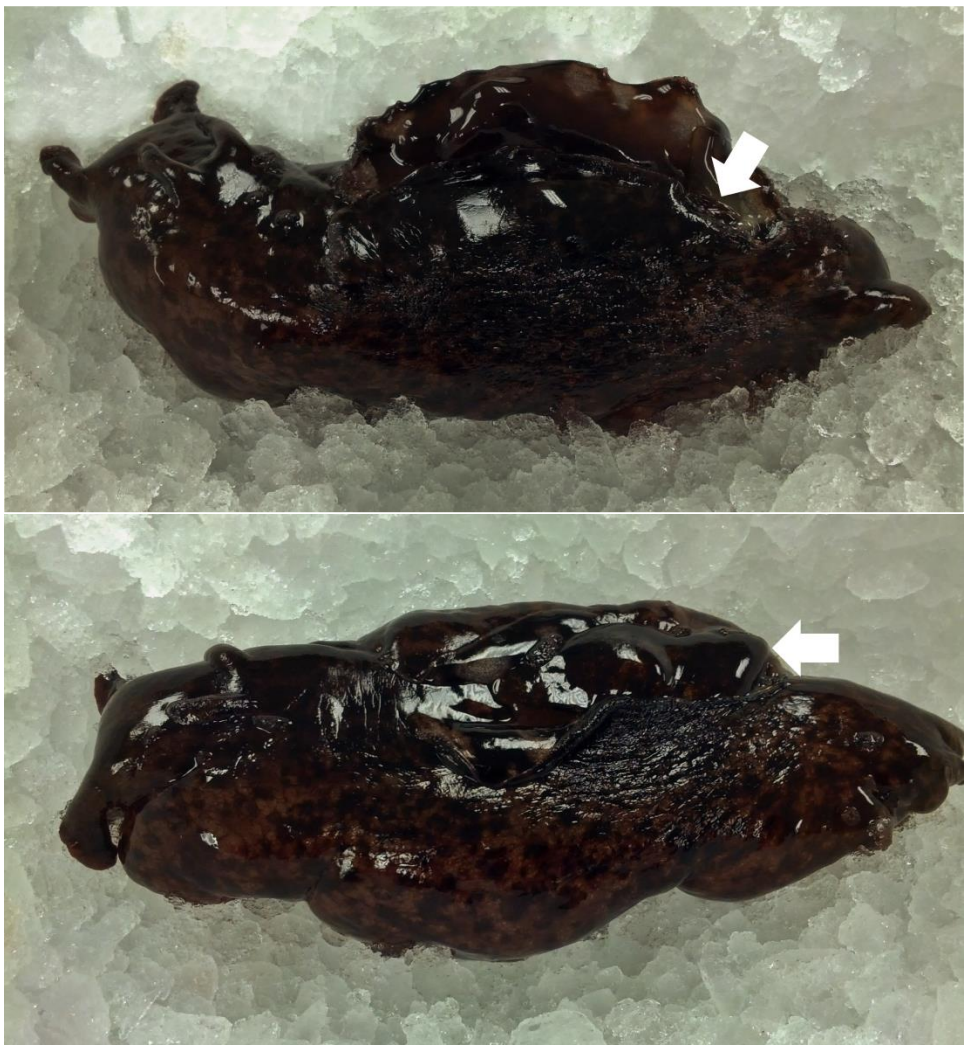

Figure 8. An *Aplysia* before (top) and after (bottom) the parapodectomy. The siphon is indicated by the white arrow in both images.

Electrode implantation: To measure the TSWR, electrical test stimuli were delivered via Teflon-coated silver wire electrodes (0.1778mm diameter; A-M Systems, Sequim, WA) implanted in one side of the parapodectomized animal's tail 3 days prior to beginning behavioral testing (Scholz and Byrne, 1987; Cleary et al., 1998). Electrodes were prepared from two 15.24cm lengths of wire. A lighter was then used to remove a small portion of the Teflon insulation from each end, and both exposed ends were trimmed to 2mm in length. A Fluke 73III multimeter (Fluke Corporation, Everett, WA) was used to ensure that there were no breaks in the Teflon insulation of prepared wires and that the resistance of each wire was less than 0.05Ω. One of the exposed ends of a prepared wire was entirely inserted into the tip of a 26G $^{3}/_{8}$ PrecisionGlide intradermal needle (Becton, Dickinson and Company, Franklin Lakes, NJ), and the wire was coiled around the outside of the bevel 7-8 times such that the length of coils matched/exceeded the length of exposed wire within. The needles were used to implant both electrodes on one side of the *Aplysia*'s tail (Fig. 9) after the animal was anaesthetized under ice for 18 min (Goldsmith and Byrne, 1993; Acheampong et al., 2012). The implantation of electrodes proceeded only if the animal did not respond to tactile stimuli, e.g., lightly brushing a rhinophore (Lechner et al., 2000a), otherwise it was placed back under ice for 3 to 5 min longer.

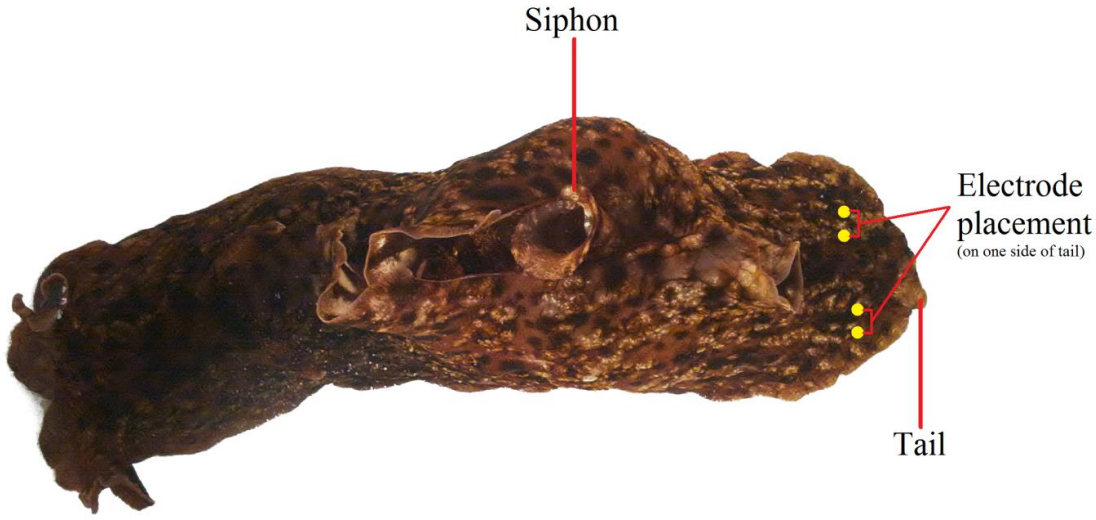

Figure 9. Placement for implantation of electrodes in either side of the tail in *Aplysia*. These electrodes comprised the test site through which the behavioral test stimuli were received.

The side of electrode implantation was determined randomly via coin toss. After successful implantation of electrodes, the *Aplysia* was transferred from its perforated plastic cage to an individual hamster ball cage (17.78cm diameter; Super Pet, IL). When in the rectangular cage, the animals may reach a corner and be forced to turn around/double back on themselves, an action that can lead to the animals crawling over their implanted electrodes, which might create tension and cause the loosening and/or loss of electrodes (Farruggella, personal observation). While in the hamster balls, the snails could move freely as the hamster ball cage rotated beneath the snail with its movements. The *Aplysia* within hamster ball cages were placed behind a plastic grating wall within the tank to minimize movement and disturbance. Approximately one year into the experiment, an additional step was added to the electrode implantation to further increase the probability of the animals retaining their implanted electrodes. Following the

implantation of both electrodes, the region of the tail where the electrodes were implanted was dried, and a small amount of Kwik-Sil Adhesive (World Precision Instruments, Sarasota, FL) was applied. Kwik-Sil is a silicone elastomer that is ideal for biomedical applications due to its rapid and low-toxicity curing that does not generate heat. After the application of Kwik-Sil, the anterior portion of the *Aplysia* was covered with ice to keep the animal anaesthetized, and the Kwik-Sil was given 18 min to cure before placing the animal into a hamster ball cage. Neither the hamster ball cages nor the application of Kwik-Sil to the tail of the animals caused any evident adverse effects to the behavior of the animals (Farruggella, personal observation).

Aplysia were not used in the behavioral testing if any of the exposed wire was visible, if the exposed wires contacted each other within the tail, or if an electrode was not adequately distant from the midline (Wainwright et al., 2002). If an animal lost one or both electrodes prior to the post-test portion of behavioral testing, post-test protocol was still followed, and the animal was treated as if the electrodes were still intact, but only feeding data were measured and analyzed (Shields-Johnson et al., 2013). If an animal lost one or both electrodes prior to beginning behavioral testing, any remaining electrode was cut near its base, and the *Aplysia* was removed from the experiment.

Drug selection: N_{ω}-nitro-L-arginine methyl ester (L-NAME; N5751, Sigma-Aldrich, Co., St. Louis, MO) was the compound used in assessing the role of NO in sensitization and sensitization-induced feeding suppression for several reasons. L-NAME is a competitive inhibitor of L-arginine for the enzyme NO synthase (NOS), effectively blocking NO synthesis (Katzoff et al., 2002; Miller et al., 2011b). L-NAME is also nontoxic to *Aplysia*; it does not affect the elicitation of appetitive and consummatory behaviors by

food stimuli (Katzoff et al., 2002). Additionally, injection of L-NAME prior to a feeding behavior training protocol – in which control animals learned that a particular food was inedible – prevented the formation of short- and long-term memory that the food item was inedible, whereas injection of D-NAME (the chemically identical enantiomer of L-NAME, which does not interact with NOS to inhibit NO production) did not (Katzoff et al., 2002; Katzoff et al., 2006). Therefore, any effects of L-NAME were due to its preclusion of NO production and did not result from any nonspecific interactions.

Behavioral testing protocol for animals in LTS training experiments (Objective 1)

The behavioral testing for the long-term sensitization experiment commenced for an animal 3 days after it had been successfully implanted with electrodes. First, the mass of the animal was measured and recorded, and then the pre-test for the TSWR began.

TSWR test: The TSWR test followed a previously established protocol, and it was used to first establish a baseline of the animal's sensitivity to the test stimuli, with changes in the duration of the TSWR from pre- to post-test used as a measure of sensitization (Scholz and Byrne, 1987; Goldsmith and Byrne, 1993; Cleary et al., 1998).

The animal was placed into a glass pedestal bowl containing 1500mL of 15°C ASW. An air bubbler was placed in the bowl to aerate the water at all times except when the animal was receiving electrical test stimuli and 1 min prior to the test stimuli. The other exposed ends of the wire electrodes were attached to the lead wire coils of a suspended electrode rig (Fig. 10) that was wired to a Variac 1504 Isolated Variable AC Line Supply (Global Specialties), which was used to deliver low-output, 20 ms-duration AC test stimuli modulated by a Pulsemaster A300 (World Precision Instruments) to the

tail of the animal. After the hook-up of electrodes, the animal was allowed to acclimate for 30 min. The threshold of the animal, which was defined as the minimum intensity of stimulation that reliably elicited an observable TSWR, was then determined by applying stimuli through the implanted electrodes in increasing 0.2mA increments.

The threshold value was measured with a Fluke 73III multimeter and recorded. The Variac was then set to double the threshold intensity to yield the test stimulus intensity that consistently produced a siphon-withdrawal response (Goldsmith and Byrne, 1993; Cleary et al., 1998; Wainwright et al., 2002). Animals were not used if they had a threshold higher than 50% of the maximum output of the Variac (4.42mA) as it would not be able to produce the test stimulus required. The animal was given 30 min to recover from threshold determination. To keep the temperature of the bowl water at 15°C, a 350mL water change with 4°C ASW was performed 10 min prior to the end of this second 30 min period. Following the 30 min recovery, baseline TSWR testing began.

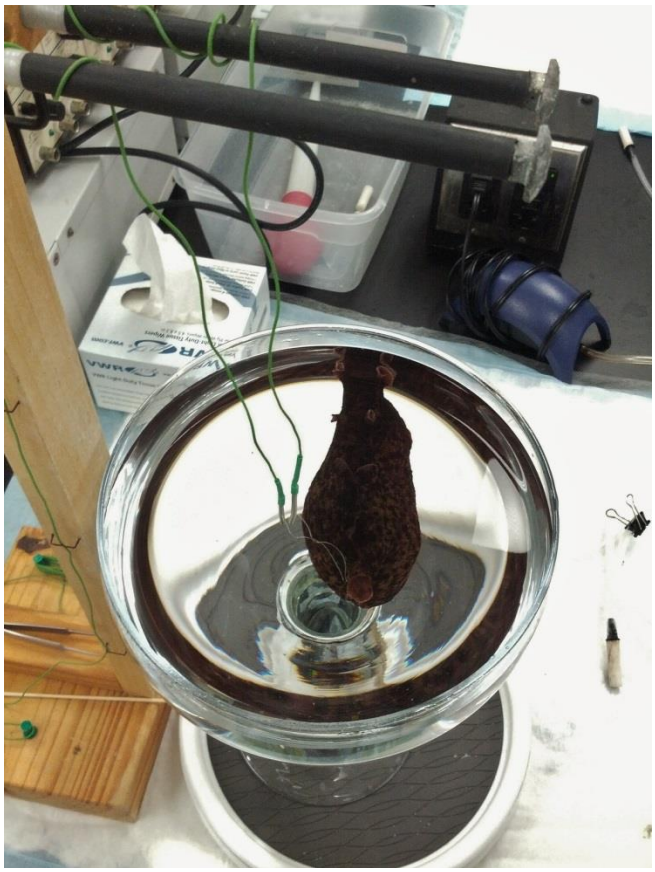

Figure 10. An *Aplysia* within the glass pedestal bowl with electrodes hooked up for TSWR testing.

The TSWR testing consisted of 5 test stimuli delivered at intervals of 10 min. After the delivery of each stimulus, the duration of the tail siphon contraction – which is a reliable index of the strength of the reflex (Goldsmith and Byrne, 1993) – was measured from the start of siphon contraction until the start of siphon relaxation (i.e., re-extension towards original position). In the event of multiple siphon withdrawal movements occurring before its return to the original position, the duration of all contractions was used. The 5 durations were averaged to determine the mean TSWR duration (Scholz and Byrne, 1987; Goldsmith and Byrne, 1993; Cleary et al., 1998). The animals must have

elicited at least 3 responses for an average to be computed, and animals were excluded if the average was greater than 10 s due to the possibility of prior sensitization (Wainwright et al., 2002). After the delivery of the final test stimulus, the animal's electrodes were unhooked from the lead wires. The post-test for TSWR duration began 24 h after the final training block and used the same test stimuli intensity as the pre-test. After an animal completed the pre- and post-test, the two mean TSWR durations were computed as a ratio (post/pre) and used as a measure of sensitization of the TSWR (Scholz and Byrne, 1987; Goldsmith and Byrne, 1993; Cleary et al., 1998).

Feeding test: The feeding pre- and post-tests were identical and occurred 30 min after the last TSWR test stimulus was delivered and the animal's electrodes were unhooked from the lead wires to eliminate any potential influence of one behavior on the other (Advokat, 1980). The protocol was the same as the aforementioned SWE feeding test. After an animal completed the feeding pre- and post-tests, the two bite counts were computed as a difference of "post – pre" (Lechner et al., 2000a; Lorenzetti et al., 2006; Acheampong et al., 2012) and used as a measure of the suppression of feeding behavior.

Injection treatment: After completion of the feeding pre-test, the *Aplysia* was placed in a colander within a plastic bowl filled with aerated 15°C ASW. The animal was injected with either an L-NAME solution or Normal ASW (i.e., vehicle) determined randomly via coin toss. The L-NAME solution was prepared daily as needed by dissolving 20mg L-NAME into 1mL of Normal ASW. Experimental injection animals were injected with 1mL of 20mg/mL L-NAME solution per 200g body mass (e.g., a 100g *Aplysia* would have been injected with 0.5mL of 20mg/mL L-NAME solution), replicating the 0.37mM concentration used by Katzoff et al. (2002). Control injection animals were injected with

1mL of vehicle per 200g body mass. The injection site was on the left side of the animal (to avoid the reproductive organs) just above the foot and perpendicular to the anterior portion of the parapodia. The *Aplysia* were injected with solution using a 26G $^{3}/_{8}$ PrecisionGlide intradermal needle.

The *Aplysia* were kept in the bowl for 1 h following the feeding test and injection before sensitization training; this was a modification to the established procedure that normally used 30 min between the end of the feeding test and the beginning of sensitization training (Acheampong et al., 2012; Shields-Johnson et al., 2013). The alteration in procedure was made based on the findings of a pilot study that was conducted to ascertain the behavioral consequences – potential long-term changes in biting behavior and duration of occurrence of spontaneous bites – of injecting a solution of L-NAME into an *Aplysia*. It is important to note that the pilot study (described subsequently) was not part of the behavioral testing protocol for animals in LTS training experiments, but the results of the pilot study influenced the experimental design of this testing protocol.

In the pilot study, a pre- and 24 h-post feeding test were performed to assess SWE-elicited consummatory feeding behavior prior to and after injection of either L-NAME or Normal ASW solutions. Following the pilot study feeding pre-test, the *Aplysia* were then injected with either L-NAME or Normal ASW solution and placed in a glass pedestal bowl with 1500mL ASW and an air bubbler. Aside from momentary contraction at the injection site upon injection, the *Aplysia* displayed no visible signs of damage nor exhibited any sensitized behavior from the injection (Farruggella, personal observation). The animals were allowed to rest for 10 min and then were observed for

50 min, with the number of spontaneous bites (i.e., consummatory feeding behavior in the absence of food stimuli) per 5 min block recorded. The time period of 50 min was selected based on observations of when spontaneous biting behavior generally diminished to zero or near zero (Fig. 11). The spontaneous bites were also observed to occur in bouts as opposed to being evenly distributed throughout each 5 min block.

Spontaneous biting behavior in animals injected with L-NAME was observed to follow a general pattern of increasing until the 20-25 min interval and then decreasing to near-zero at the 45-50 min interval (Fig. 11). The average amount of spontaneous bites elicited by animals injected with L-NAME solution was significantly different than those elicited by animals injected with Normal ASW at the 20-25 minute interval (L-NAME: 8.33±2.64 bites, n=9; Control: 0.00±0.00 bites, n=4; $p < 0.05$, U=4.000; Mann-Whitney U test; Fig. 11) but not at the 45-50 min interval (L-NAME: 2.56±1.25 bites, n=13; Control: 0.00±0.00 bites, n=4; $p = 0.158$, U=10.000; Mann-Whitney U test; Fig. 11). This information and observations proved essential for the experimental design of this project and were the causes for the modification of the pre-established protocol. The time between the LTS feeding pre-test and LTS training was increased from 30 min to 1 h to allow spontaneous biting behavior to decrease to baseline/near-zero amounts. This procedural modification ensured that spontaneous biting behavior was at its minimum during sensitization training in order to prevent the possible formation of associative memory between the sensitization training shocks and biting.

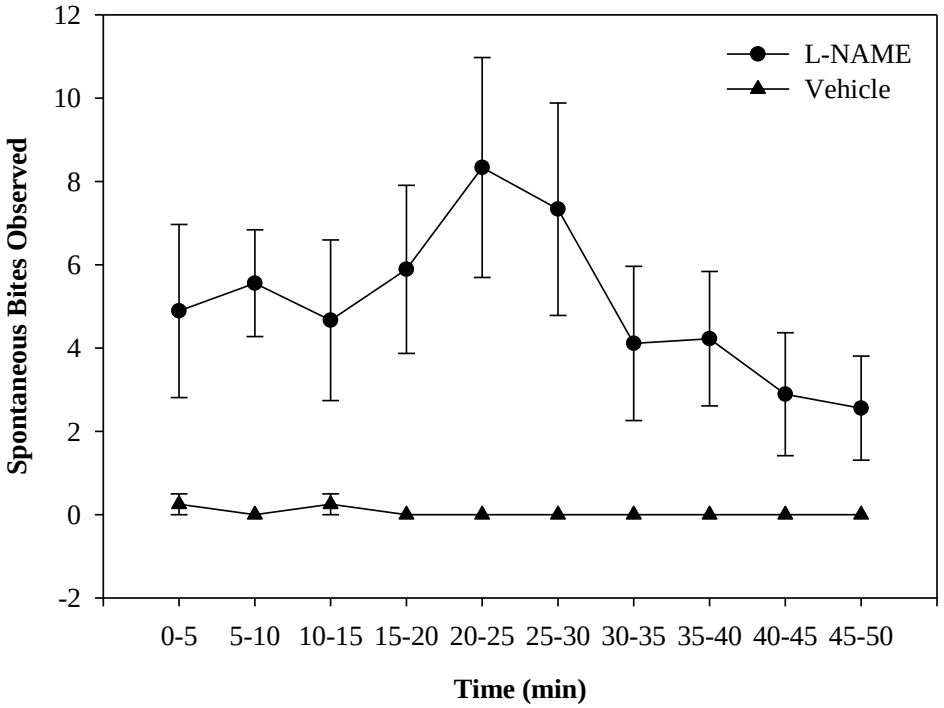

Figure 11. Spontaneous biting behavior of *Aplysia* injected with L-NAME solution (n=9) compared to those injected with vehicle (n=4). In this figure and all subsequent figures, the error bars indicate standard error of measurement (SEM).

The other important finding of the pilot study was derived from the feeding post-test that was conducted on the same animals 24 h after injection. The bite differences were calculated (post-test bites – pre-test bites) and compared between L-NAME treatment animals and Normal ASW treatment animals (Fig. 12). Despite using a different food stimulus than Katzoff et al. (2002) – pure chemical stimulus SWE rather than stimulation of the animal's lips with seaweed – it was similarly observed that there was no significant difference in feeding parameters measured between the L-NAME and Normal ASW injected treatment groups (mean bite difference; L-NAME: -3.00±5.41 bites, n=8; Vehicle: -5.00±3.22 bites, n=3; $p = 0.497$, U=8.000; Mann-Whitney U test; Fig. 12). This information showed that injecting *Aplysia* with either L-NAME or vehicle

solutions caused no long-term alterations in feeding behavior that would affect the feeding tests in the LTS experiment.

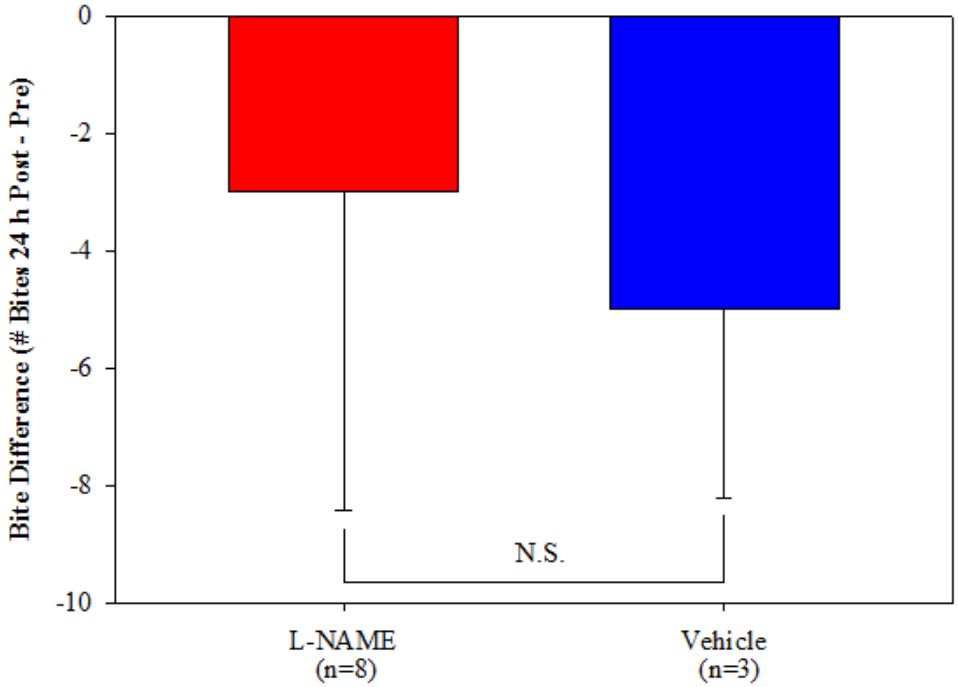

Figure 12. Difference in SWE-elicited biting behavior of post- and pre-tests of *Aplysia* injected with L-NAME solution compared to those injected with vehicle. In this figure and all subsequent figures, N.S. means no significance.

Long-term sensitization training: The training protocol that was used has been shown to produce LTS (Scholz and Byrne, 1987; Goldsmith and Byrne, 1993; Cleary et al., 1998; Wainwright et al., 2002; Khabour et al., 2004) as well as a concomitant suppression of feeding behavior (Acheampong et al., 2012; Shields-Johnson et al., 2013) detectable for at least 24 h. The training occurred 1 h after the completion of the feeding pre-test and was performed by a tester other than the experimenter who performed the pre- and post-tests. The training treatment was determined randomly via coin toss by the tester who would perform the protocol (i.e., the trainer). Untrained animals were handled

identically to trained, but they did not receive sensitization training stimuli (Wainwright et al., 2002). Training consisted of 4 blocks of sensitizing stimuli delivered at intervals of 30 min, and each training block consisted of a 10 s duration train of 10 high-output AC training stimuli (500ms duration, 1 Hz, 60mA; Fig. 13). The training stimuli were diffusely delivered to the body wall of the animal on the side ipsilateral to electrode implantation (Fig. 14), an area that is outside of the tail sensory neuron receptive fields (Walters et al., 1983a), and similarly used the Variac/Pulsemaster instrumentation combination. The training wand used to apply the training stimuli to the *Aplysia* body wall contains two 24 gauge silver electrodes.

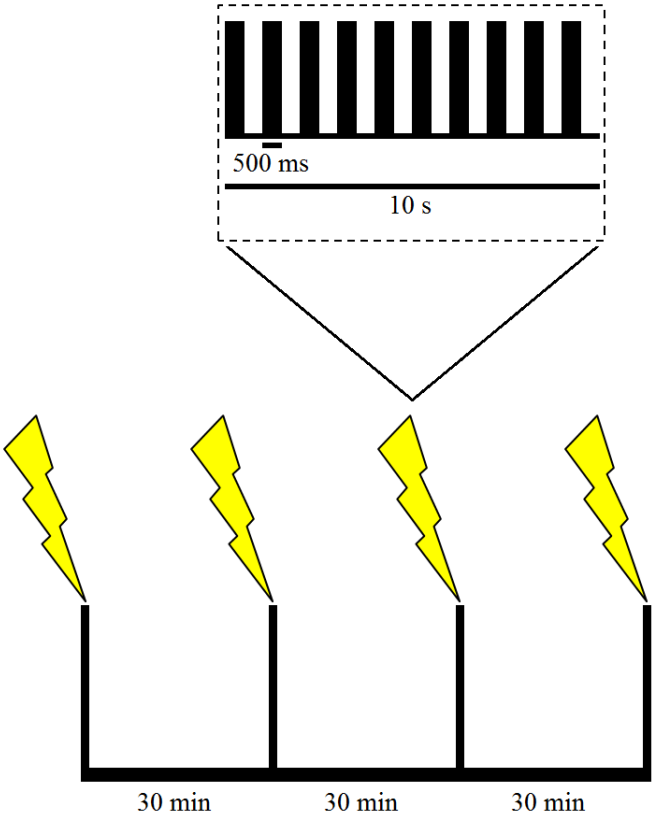

Figure 13. Protocol for LTS training. Yellow bolts indicate sensitization training stimuli.

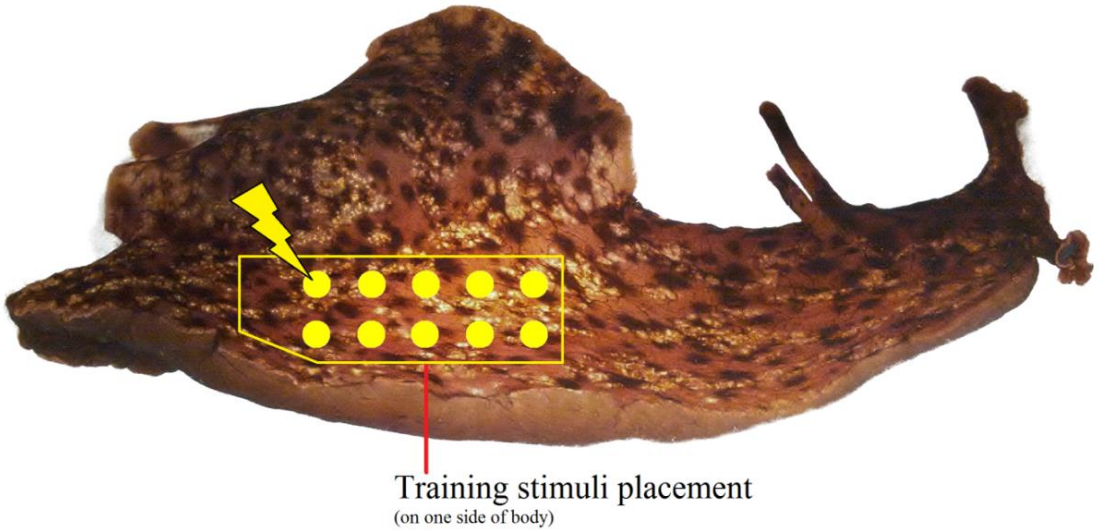

Figure 14. General area on *Aplysia* body wall for delivery of sensitizing training stimuli. The training stimuli (yellow bolt) were diffusely applied to avoid damaging the skin.

In response to proper delivery of the training stimuli, the *Aplysia* reliably demonstrated defensive responses such as release of ink, release of opaline, and increase in escape locomotion (Carew et al., 1981; Scholz and Byrne, 1987; Goldsmith and Byrne, 1993; Cleary et al., 1998; Wainwright et al., 2002). These defensive responses were qualitatively recorded by the tester who transferred the animal to a clean bowl of ASW after each training stimulus. Animals were excluded if they did not release ink or opaline in response to at least one of the training stimuli. After training, animals were returned to the tank.

TSWR and Feeding post-tests: The first TSWR measurement of the post-test occurred 24 h after the 4th and final training block. The LTS behavior pre- and post-tests were identical, except threshold was not re-determined during the post-test, nor was the animal injected a second time following the feeding post-test.

Behavioral testing protocol for animals in single-trial training experiments (Objective 2)

The behavioral testing for the single-trial sensitization training experiment examined only *Aplysia* feeding behavior; therefore, this experiment utilized naïve animals that required no experimental preparation other than the universal deprivation of food for 48 h prior to the experiment. First, the mass of the animal was measured and recorded. An initial SWE feeding test was conducted to establish the pre-test baseline.

Injection treatment: After completion of the feeding pre-test, the *Aplysia* was placed in a colander within a plastic bowl filled with aerated 15°C ASW. The animal was injected with either an L-NAME or vehicle (Normal ASW) solution, which was determined randomly via coin toss, and was performed in an identical manner as in the aforementioned long-term behavior experiment (Objective 1). The animal was kept in the bowl for 1 h following the feeding test and injection before sensitization training; this was a modification to the established procedure that normally used 30 min between the end of the feeding test and the beginning of sensitization training (Acheampong et al., 2012; Shields-Johnson et al., 2013). The alteration in procedure was made based on the findings of the aforementioned pilot study.

Single-trial sensitization training: The single-trial training protocol that was used has been shown to produce sensitization detectable at 15 min and 2 h post-training but not at 24 h post-training (Acheampong et al., 2012). This single-trial training protocol has also been shown to concurrently induce a suppression of feeding behavior detectable at the same points in time (Acheampong et al., 2012). The training occurred 1 h after the completion of the feeding pre-test and was performed by a tester other than the

experimenter who performed the pre- and post-tests. The training treatment was determined randomly via coin toss by the trainer. Training consisted of a single block of sensitizing stimuli that consisted of a 10 s duration train of 10 high-output AC training stimuli (500ms duration, 1 Hz, 60mA), which was identical to LTS training (Fig. 13) except it was only a single block of sensitizing stimuli rather than 4 spaced blocks. The training stimuli were diffusely delivered to the body wall of the animal on one side determined randomly via coin toss by the trainer, and similarly used the Variac/Pulsemaster instrumentation combination. The training wand used to apply the training stimuli was identical to that used for LTS training experiments (Objective 1). Untrained animals were handled identically to trained, but they did not receive sensitization training stimuli (Wainwright et al., 2002). To keep the temperature of the bowl water at 15°C, a 350mL water change with 4°C ASW was performed 1 h after training.

Feeding post-tests: SWE feeding post-tests were conducted at time points 15 min, 2 h, and 24 h post-training. After an animal completed the pre- and post-tests, the bite counts were computed as differences for the three post-training time points (post – pre) and used as a measure of sensitization and suppression of feeding behavior (Lechner et al., 2000a; Lorenzetti et al., 2006; Acheampong et al., 2012).

Dissection and electrophysiology protocol (Objective 3)

In this series of experiments, the role of NO signaling was examined *in vitro* at the level of the feeding neural circuit. *Aplysia* were dissected and prepared for intracellular recording after they elicited bites when their lips were brushed with a piece

of seaweed – which is known to elicit biting behavior (Schwarz and Susswein, 1986; Botzer et al., 1998) – to confirm the functionality of the feeding neural circuit.

Aplysia to be dissected were first anaesthetized by injecting a volume (mL) of isotonic $MgCl_2$ equal to 50% of the animal's mass into the hemocoel through the foot (Scholz and Byrne, 1987). The dissection, which sacrificed the animal, proceeded only after the animal ceased to respond to tactile stimuli, e.g., lightly brushing a rhinophore (Lechner et al., 2000a). The entire buccal mass was excised and pinned in a Sylgard-coated dish filled with high divalent cation concentration ASW (Hi-Di ASW; Fig. 15). The Hi-Di ASW served to suppress polysynaptic neural activity during manipulation of the buccal ganglion by competing for ion channels (Byrne et al., 1978; Nargeot et al., 1997). The Hi-Di ASW was comprised of 210mM NaCl, 10mM KCl, 145mM $MgCl_2$, 20mM $MgSO_4$, 33mM $CaCl_2$, and 10mM HEPES, with the pH adjusted to 7.5 using NaOH (Mozzachiodi et al., 2003).

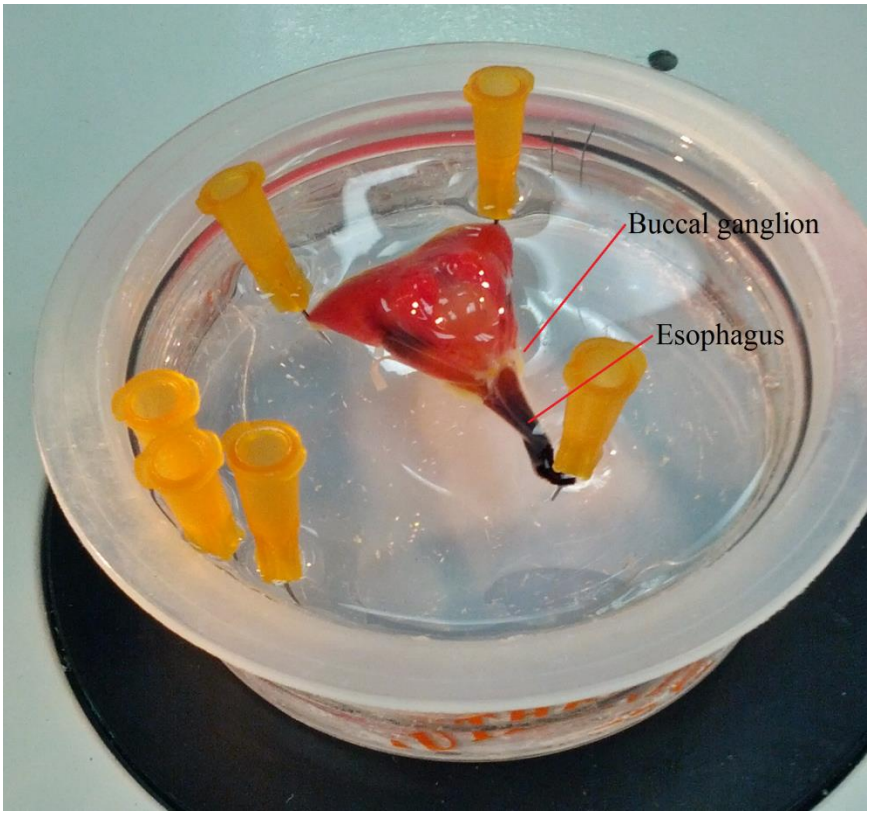

Figure 15. An excised and pinned buccal mass.

The buccal ganglion and the third branch of the peripheral buccal nerves (Bn.2,3) were cut away from the buccal mass and transferred to a Sylgard-coated petri dish containing a concentric inner chamber filled with Hi-Di ASW. The volume of the inner recording chamber was set to 1.5mL and was maintained throughout the duration of experiment. The buccal ganglion and attached nerves were pinned relatively tautly to the dish within the inner chamber with the rostral side facing upwards, and Bn.2,3 was drawn away from the ganglion, pinned outside of the inner chamber, and isolated from the Hi-Di ASW bath using petroleum jelly to form a well (Figs. 16, 17) (Nargeot et al., 1997; Mozzachiodi et al., 2003). The sheathing of the hemi-ganglion contralateral to the isolated Bn.2,3 was delicately cut away and removed to expose the soma of neuron B51

(Fig. 17). Bipolar electrodes were placed on both sides of the isolated Bn.2,3 after removing the Hi-Di ASW within the well, and the well was filled in with petroleum jelly. These electrodes were used for monotonic electrical stimulation that mimicked stimulation by food to elicit fictive feeding behavior in the form of B51 BMPs (Nargeot et al., 1997; Mozzachiodi et al., 2008). After desheathing was completed and the bipolar electrodes were in place, the Hi-Di ASW in the inner chamber was gradually washed out with and exchanged for Normal ASW in 10 exchanges of 750µL, and the ganglion was allowed to rest for 30 min to re-enable synaptic activity before taking measurements (Nargeot et al., 1999a; Shields-Johnson et al., 2013).

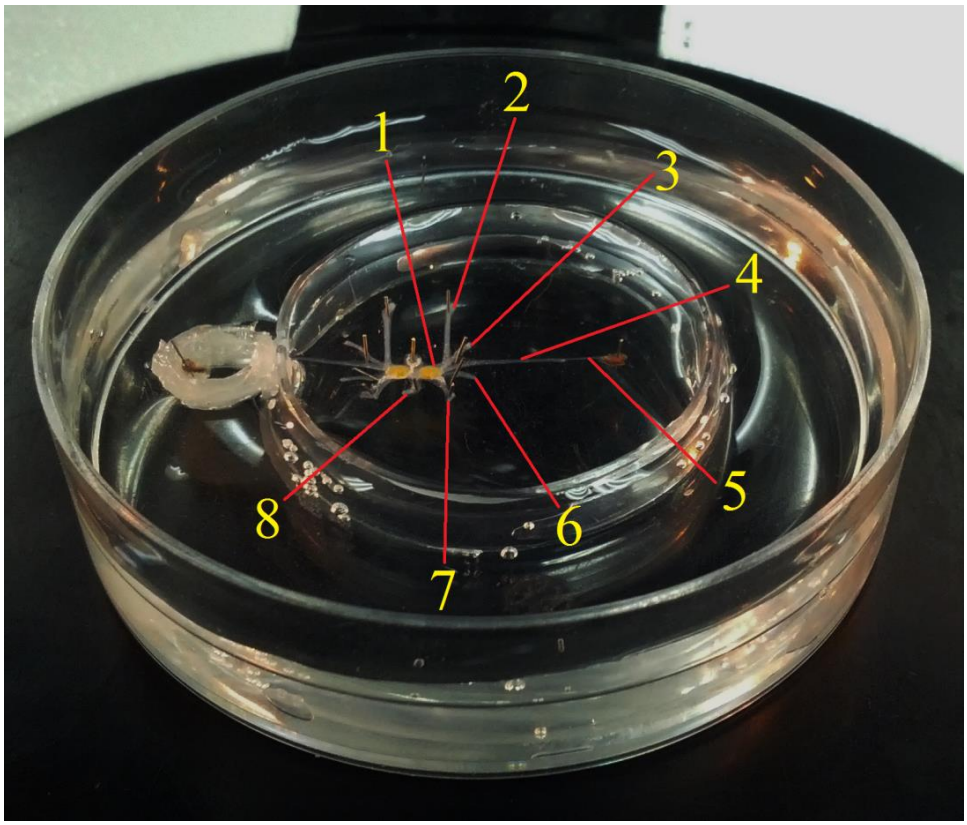

Figure 16. An excised and pinned buccal ganglion (1). Included are the cerebral buccal connective nerve (2), buccal nerve 3 (3), buccal nerve 2 (4), third branch of buccal nerve 2 (5), buccal nerve 1 (6), esophageal nerve (7), and radular nerves (8).

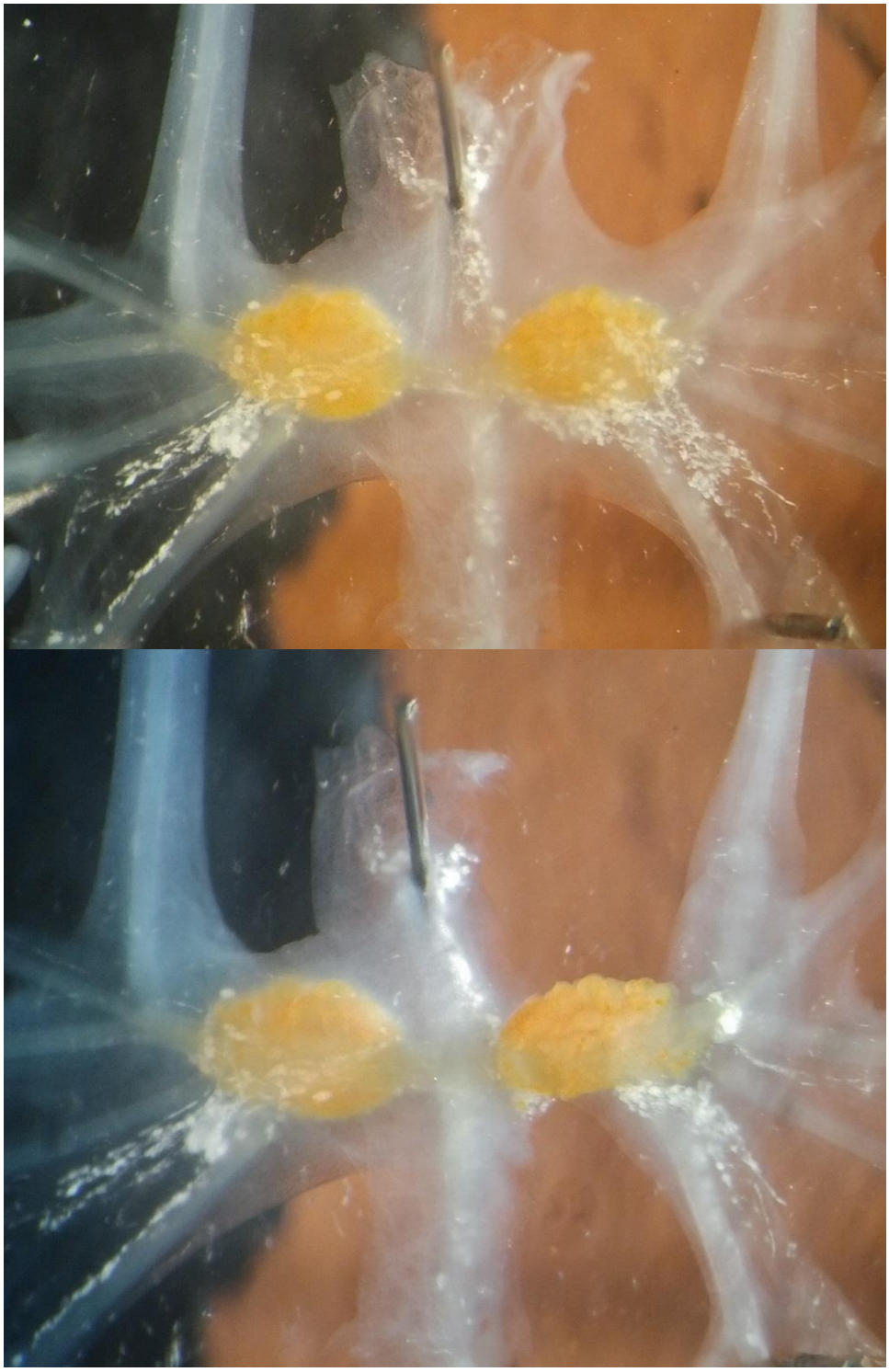

Figure 17. A closer view of an excised and pinned buccal ganglion, with tissue sheathing (top) and desheathed (bottom).

The temperature of the bath was maintained at 15°C using a BTC-100 (Bioscience Tools, San Diego, CA) temperature controller in conjunction with a VWR Variable Flow Mini-pump (VWR International, Radnor, PA). A standard two-electrode current clamp technique was used for intracellular recording and measurement of intrinsic properties and activity of B51 (Fig. 18) (Nargeot et al., 1999a). Fine-tipped glass microelectrodes (resistance 10-12MΩ) were made using a Micropipette Puller Model P-97 (Sutter Instruments, Novato, CA) and were filled with 3M potassium acetate solution (Scholz and Byrne, 1987; Shields-Johnson et al., 2013). Silver wire electrodes (not coated) held by micromanipulators were inserted into the microelectrodes for use in intracellular stimulation and recording (0.254 mm diameter, 30 gauge, A-M Systems).

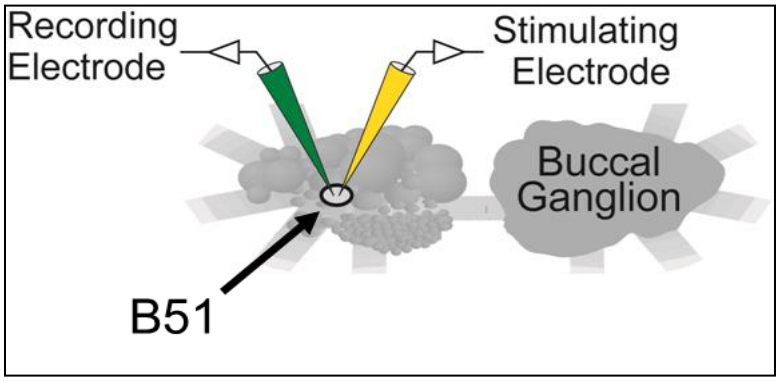

Figure 18. Two-electrode current clamp setup for electrophysiology. Image provided by Dr. Mozzachiodi.

LabChart 7 software (ADInstruments, Dunedin, New Zealand) was used to visualize and manipulate neuronal activity. A PowerLab 8/30 (ADInstruments) was used for data acquisition and networking instrumentation. An IX2-700 Dual Intracellular

Preamplifier (Dagan Corporation, Minneapolis, MN) modulated with a Pulsemaster A300 was used for stimulation, current injection, and facilitation of cellular protocols.

Measurement of B51 properties and activity: After the Hi-Di ASW was exchanged with Normal ASW and the ganglion was allowed to rest for 30 min, B51 membrane properties and cellular activity were measured. First, B51 in the desheathed, contralateral hemi-ganglia was located based on its relative size, relative location (Figs. 19, 20), and characteristic plateau potential activity (Fig. 3) in response to monotonic electrical stimulation of Bn.2,3 (Plummer and Kirk, 1990; Nargeot et al., 1997, 1999a, b; Mozzachiodi et al., 2008). B51 was impaled with two intracellular microelectrodes (recording and stimulating; Fig. 15) and allowed to recover for 5 min.

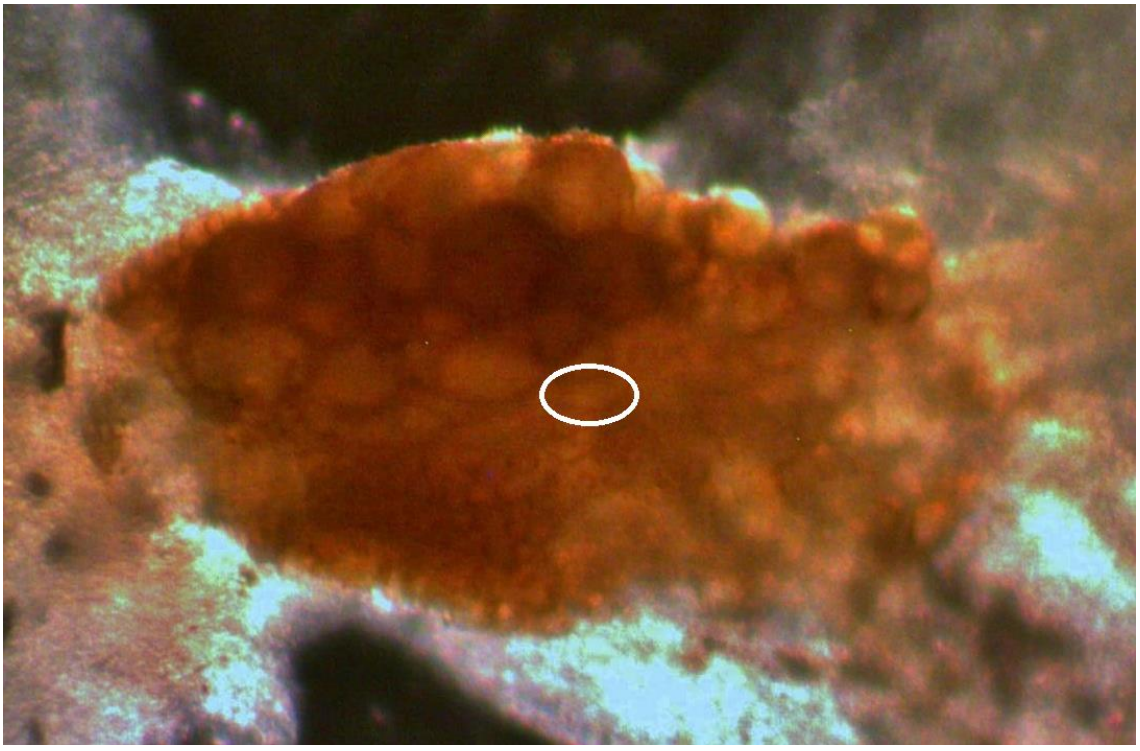

Figure 19. A desheathed buccal hemi-ganglion contralateral to the isolated Bn.2,3. Neuron B51 is within the white ellipse.

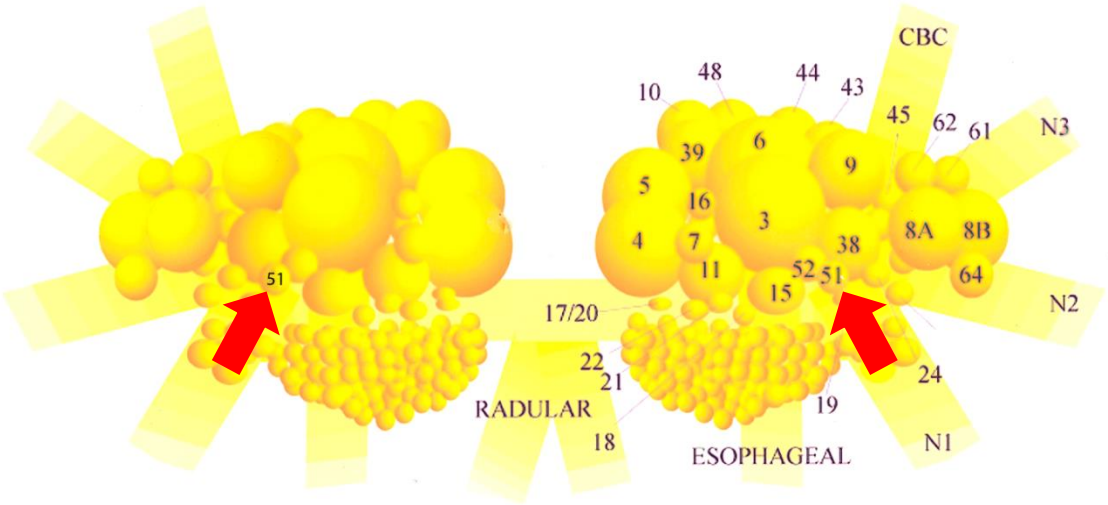

Figure 20. The mapped rostral surface of the buccal ganglion. Neuron B51 is indicated in each hemi-ganglia with a red arrow. Adapted from Church and Lloyd (1991).

Resting membrane potential (V_m) was determined from the readout (Fig. 21 A) of the recording electrode after the 5 min recovery time. Input resistance (R_{in}) and burst threshold (BT) were obtained while B51 was current-clamped at -60mV by injecting DC through the stimulating electrode (Nargeot et al., 1999a, b; Lorenzetti et al., 2006; Mozzachiodi et al., 2008). R_{in} (in MΩ) was determined by injecting 5 s of 5nA hyperpolarizing current (Fig. 21 B), measuring the change in voltage from baseline before pulse to just before pulse ends, and solving Ohm's Law (I = V/R) for resistance. BT was determined by injecting 5-s pulses of depolarizing current with 10-s intervals, beginning at 5nA (Fig. 21 C) and increasing in 1nA increments to 30nA until burst activity was elicited that outlasted the stimulatory pulse (Fig. 21 D) (Nargeot et al., 1999a; Lorenzetti et al., 2006; Mozzachiodi et al., 2008). B51 neurons that did not burst between 5 and 30nA were assigned the maximum burst threshold of 30nA (Shields-Johnson et al., 2013). If a spontaneous motor pattern occurred while measuring the

intrinsic properties of B51, recording was halted and then resumed 1 min after the spontaneous BMP had ended (Mozzachiodi et al., 2008). The current clamp was removed and the ganglion was allowed to rest for 10 min following the completion of the pre-bolus intrinsic properties measurements. At the end of the 10 min, 15µL was removed from the inner recording chamber, and an experimental bolus (15µL) consisting of L-NAME/SNAP or vehicle (see below) was injected near the ganglion and mixed via gentle pipetting without physically disturbing the neurons. The ganglion was given 3 min to rest following the bolus application, after which the intrinsic properties were re-measured.

Figure 21. Representative intracellular recordings from B51 illustrating the measurements of V_m (A), R_{in} (B), and BT (C, D).

Objective 3.1 protocol: In addition to the aforementioned intrinsic properties (V_m, R_{in}, BT), the duration of the BT plateau potential was also measured. The bolus consisted of either 0.037M L-NAME solution (such that when injected into the chamber, the concentration became 0.37mM as previously used by Katzoff et al. (2002)) or Normal ASW (i.e., vehicle control), which was determined randomly via coin toss. After

completion of post-bolus intrinsic properties measurements (including BT duration), the current clamp was removed and the ganglion was allowed to rest for 10 min.

The monotonic electrical stimulation of Bn.2,3 was delivered at 4 Hz, 10V pulse height, and 0.5 ms pulse width. This stimulation activated the feeding CPG and evoked fictive feeding patterns comprised of the neuronal correlates of feeding behavior (Nargeot et al., 1997, 1999a; Mozzachiodi et al., 2008). The number of B51 plateau potentials lasting at least 1 s (Fig. 22), the number of B51 sub-threshold depolarizations (Fig. 22), and the latency from the start of Bn.2,3 stimulation to the first plateau potential were assessed for 5 min of constant monotonic stimulation (Nargeot et al., 1999a, b; Mozzachiodi et al., 2008). The plateau potentials were found to be the neuronal correlates iBMPs, and the sub-threshold depolarizations were found to be the correlates of eBMPs (Nargeot et al., 1999a, b). The occurrence of spontaneous plateau potentials and spontaneous sub-threshold depolarizations in the absence of artificial intra- or extracellular stimulation were also quantified pre- and post-bolus for both treatments.

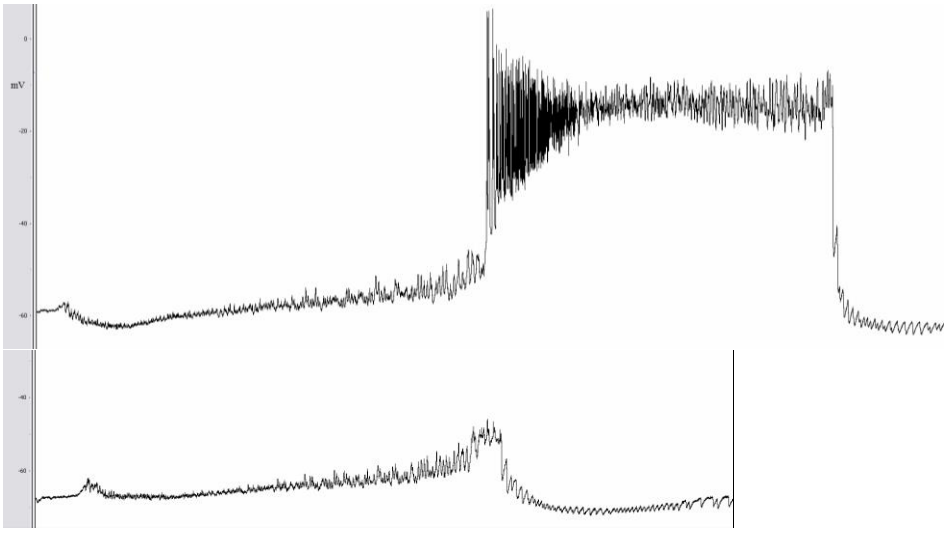

Figure 22. Representative intracellular recordings from B51 illustrating a plateau potential (top) and sub-threshold depolarization (bottom).

Objective 3.2 protocol: This objective tested the effect of SNAP on B51 membrane properties, which were measured as previously described. The bolus consisted of either a SNAP (experimental) or Normal ASW-dimethyl sulfoxide (DMSO; vehicle control) solution, which was determined randomly via coin toss. This SNAP treatment experiment was selected as an addendum to the L-NAME-focused Objective 3.1 because SNAP donates NO, adding exogenous NO to the surrounding solution, through the redox-mediated release of its nitroso group that is part of the *S*-nitrosothiol section of the compound attached to the amino acid derivative. In previous experiments, the injection of SNAP *in vivo* increased the concentration of NO beyond its normal tonic levels and consequently increased its inhibitory effect, causing a reduction in feeding behavior in the presence of food stimuli (Miller et al., 2011b). Treatment with SNAP has also been shown to negate the feeding-related memory formation-blocking effects of NO synthase inhibitors (Katzoff et al., 2002) and to enable feeding-related memory formation from normally sub-threshold stimuli (Katzoff et al., 2006).

The SNAP solution was comprised of SNAP (N7927, Life Technologies, Eugene, OR), DMSO (D12345, Life Technologies), and Normal ASW, and was prepared in a manner such that the final concentration in the inner chamber was 100μM SNAP and 0.1% by volume DMSO. The vehicle solution was comprised of DMSO and Normal ASW, and was prepared in a manner such that the final concentration in the inner chamber was 0.1% by volume DMSO.

Statistical analysis

All statistical analyses and visualization of data were accomplished using SigmaPlot 11.0 (Systat Software, Inc., San Jose, CA). Statistical significance was set at $p < 0.05$. For raw data transformation prior to graphing, ratios were computed as "post/pre", differences as "post – pre", and percent changes as "(post – pre)/pre x 100" where relevant and useful for comparisons. All data illustrated by figures was represented as "mean ± standard error of the mean".

For the long-term sensitization behavioral testing data (Objective 1), the change in TSWR durations was expressed as a ratio of "post/pre", and the change in number of bites was expressed as a difference of "post – pre." These values were compared among the different experimental groups of L-NAME-trained, L-NAME-untrained, vehicle-trained, and vehicle-untrained *Aplysia*. The statistical tests employed were based on the normality of distribution of the data. Data that was normally distributed was analyzed with parametric statistics using a One-way ANOVA and Student-Newman-Keuls *post hoc* test if revealed to be significant (Day and Quinn, 1989). Data that was not normally distributed was analyzed with nonparametric statistics using the Kruskal-Wallis H test (Mozzachiodi et al., 2003; Mozzachiodi et al., 2008; Acheampong et al., 2012) and Dunn's *post hoc* test if revealed to be significant (Day and Quinn, 1989).

For the single-trial training behavioral testing data (Objective 2), the change in number of bites was expressed as a difference for each of the time points ("15 min post – pre", "2 h post – pre", and "24 h post – pre"). These values were compared among the

four experimental *Aplysia* groups in each of the three time categories and analyzed using the same statistical protocol as the LTS behavioral data.

For the *in vitro* L-NAME bolus experiments (Objective 3.1), the changes in B51 properties (including BT plateau potential duration) were expressed as a percent change of "(post – pre)/pre x 100" and were compared between L-NAME and vehicle groups using the Mann-Whitney U test. Quantified B51 activity (including stimulated plateau potential durations) was compared between L-NAME and vehicle groups using the Mann-Whitney U test. Spontaneous B51 activity, i.e., activity of B51 generated in the absence of artificial intra- or extracellular stimulation, was also quantified. Spontaneous plateau potentials and sub-threshold depolarizations were expressed as differences in number "post-bolus – pre-bolus" and were compared between L-NAME and vehicle groups using the Mann-Whitney U test.

For the *in vitro* SNAP bolus experiments (Objective 3.2), the changes in B51 properties were expressed as a percent change of "(post – pre)/pre x 100" and were compared between SNAP and vehicle groups using the Mann-Whitney U test.

RESULTS

Effects of L-NAME on behavioral changes induced by LTS training

The purpose of this experiment was to examine the role of NO in the LTS training-induced changes to the TSWR and feeding behavior. For the TSWR, the Kruskal-Wallis H test revealed an overall significant difference among the groups ($p < 0.05$). The TSWR was significantly enhanced by LTS training only in vehicle-trained animals (Fig. 23). Pairwise *post hoc* comparisons (Dunn's test) showed that vehicle-trained animals experienced significant TSWR enhancement (i.e., LTS; TSWR ratio; 2.69±0.51, n=16) compared to L-NAME-trained (TSWR ratio; 0.89±0.10, n=12; $p < 0.05$, Q=2.716), L-NAME-untrained (TSWR ratio; 1.06±0.25, n=14; $p < 0.05$, Q=2.862), and vehicle-untrained (TSWR ratio; 1.09±0.36, n=9; $p < 0.05$, Q=2.834; Fig. 23). There were no significant differences between the other groups: L-NAME-trained vs. L-NAME-untrained ($p > 0.05$, Q=0.027); L-NAME-trained vs. vehicle-untrained ($p > 0.05$, Q=0.326); L-NAME-untrained vs. vehicle-untrained ($p > 0.05$, Q=0.312; Fig. 23). This experiment demonstrated that blocking NO signaling by inhibiting its synthesis prevented the occurrence of LTS of the TSWR.

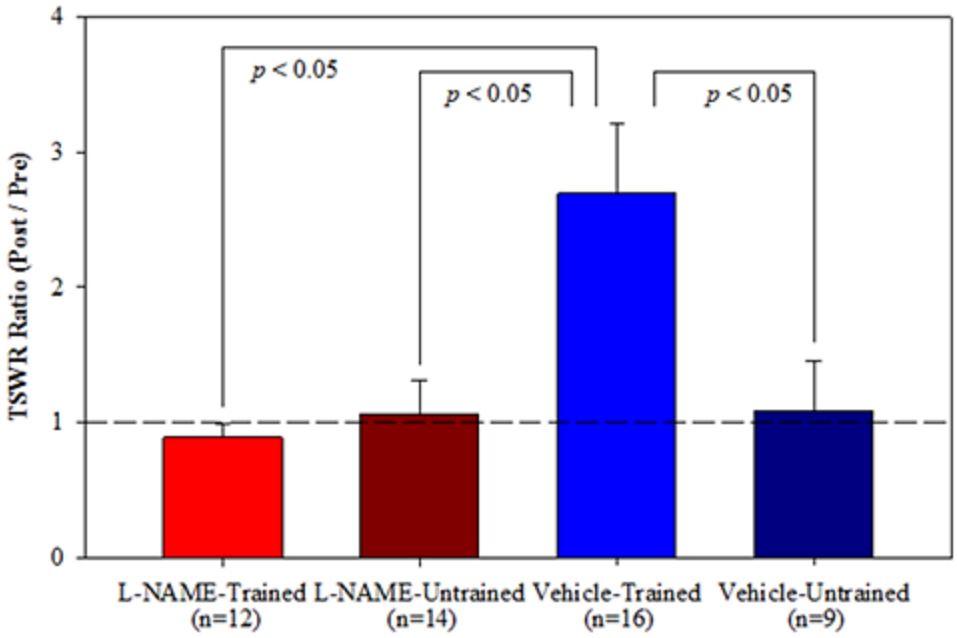

Figure 23. The effect of L-NAME on the LTS of the TSWR. The dotted line indicates a TSWR ratio of 1. A TSWR ratio greater that 1 is indicative of occurred sensitization.

The effects of L-NAME were also analyzed on the training-induced changes in feeding behavior in the same group of animals used above to examine LTS. The One-way ANOVA revealed an overall significant difference among the groups ($p < 0.05$). Feeding behavior was significantly suppressed by LTS training only in vehicle-trained animals (Fig. 24). Pairwise *post hoc* comparisons (Student-Newman-Keuls test) showed that vehicle-trained animals experienced significant feeding suppression (bite difference; -8.84±2.27, n=19; F=5.810, Power = 0.891) compared to L-NAME-trained (bite difference; -0.36±1.78, n=11; $p < 0.05$, q=4.051), L-NAME-untrained (bite difference; -1.25±1.15, n=16; $p < 0.05$, q=4.051), and vehicle-untrained animals (bite difference; 1.92±2.56, n=12; $p < 0.05$, q=5.282; Fig. 24). There were no significant differences between the other groups: L-NAME-trained vs. L-NAME-untrained ($p = 0.773$,

q=0.410); L-NAME-trained vs. vehicle-untrained (*p* = 0.487, *q*=0.989); L-NAME-untrained vs. vehicle-untrained (*p* = 0.542, *q*=1.501; Fig. 24). This experiment demonstrated that blocking NO signaling prevented the LTS training-induced suppression of feeding behavior.

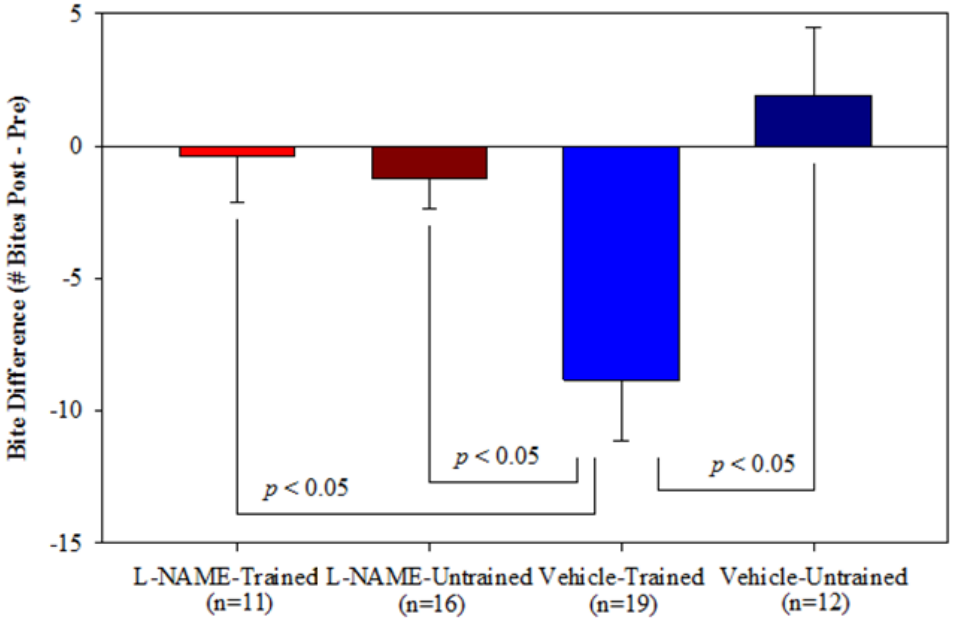

Figure 24. The effect of L-NAME on LTS training-induced suppression of feeding behavior 24 h after training.

Effects of L-NAME on changes in feeding behavior induced by single-trial training

The purpose of this experiment was to examine the role of NO in the single-trial training-induced changes to feeding behavior. At 15 min post-training, the One-way ANOVA revealed an overall significant difference among the groups ($p < 0.05$). Feeding behavior was significantly suppressed by single-trial training only in vehicle-trained animals at the 15 min post-test (Fig. 25). Pairwise *post hoc* comparisons (Student-Newman-Keuls test) showed that vehicle-trained animals experienced

significant feeding suppression (bite difference; -8.53±2.11, n=15; F=4.983, Power = 0.810) compared to L-NAME-trained (bite difference; 1.54±2.77, n=13; $p < 0.05$, q=4.256), L-NAME-untrained (bite difference; -0.91±1.64, n=11; $p < 0.05$, q=3.075), and vehicle-untrained animals (bite difference; 3.31±2.90, n=13; $p < 0.05$, q=5.003; Fig. 25). There were no significant differences between the other groups: L-NAME-trained vs. L-NAME-untrained ($p = 0.502$, q=0.957); L-NAME-trained vs. vehicle-untrained ($p = 0.612$, q=0.722); L-NAME-untrained vs. vehicle-untrained ($p = 0.479$, q=1.648; Fig. 25). This experiment demonstrated that blocking NO signaling prevented the single-trial training-induced suppression of feeding behavior at 15 min post-training.

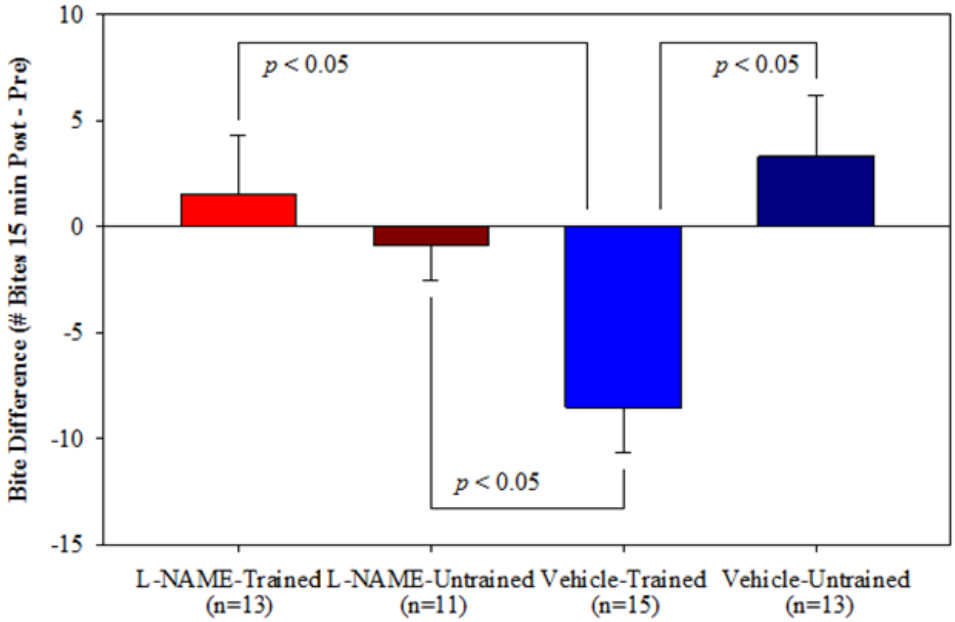

Figure 25. The effect of L-NAME on the suppression of feeding behavior induced by single-trial training 15 min after training.

At 2 h post-training, the Kruskal-Wallis H test revealed an overall significant difference among the groups ($p < 0.05$). Feeding behavior was also significantly

suppressed by single-trial training only in vehicle-trained animals at the 2 h post-test (Fig. 26). Pairwise *post hoc* comparisons (Dunn's test) showed that vehicle-trained animals experienced significant feeding suppression (bite difference; -10.25±1.12, n=16) compared to L-NAME-trained (bite difference; -0.58±2.53, n=12; $p < 0.05$, Q=3.479), L-NAME-untrained (bite difference; -1.36±1.83, n=11; $p < 0.05$, Q=3.054), and vehicle-untrained animals (bite difference; -2.54±2.32, n=13; $p < 0.05$, Q=2.995; Fig. 26). There were no significant differences between the other groups: L-NAME-trained vs. L-NAME-untrained ($p > 0.05$, Q=0.317); L-NAME-trained vs. vehicle-untrained ($p > 0.05$, Q=0.525); L-NAME-untrained vs. vehicle-untrained ($p > 0.05$, Q=0.190; Fig. 26). This experiment demonstrated that blocking NO signaling also prevented the single-trial training-induced suppression of feeding behavior at 2 h post-training.

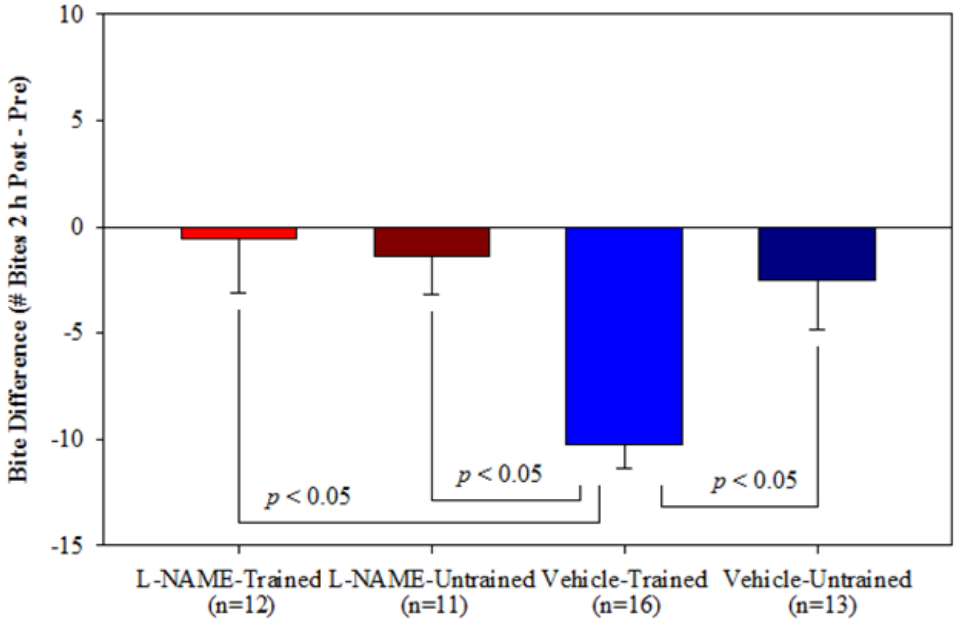

Figure 26. The effect of L-NAME on the suppression of feeding behavior induced by single-trial training 2 h after training.

At 24 h post-training, the Kruskal-Wallis H test revealed that there was no overall significant difference among the groups (p = 0.322). There was no significant evidence of feeding suppression induced by single-trial training in any of the groups after 24 h (L-NAME-trained: 1.90±2.19, n=10; L-NAME-untrained: -2.33±1.64, n=9; vehicle-trained: -1.92±1.70, n=12; vehicle-untrained: -0.15±3.07, n=13; Fig. 27). No significant difference was expected at the 24 h time point, as the concomitant suppression of feeding produced by single-trial training was not detectable after 24 h in previous studies (Acheampong et al., 2012). This experiment also demonstrated that the animals treated with L-NAME were not experiencing any adverse effects from the drug.

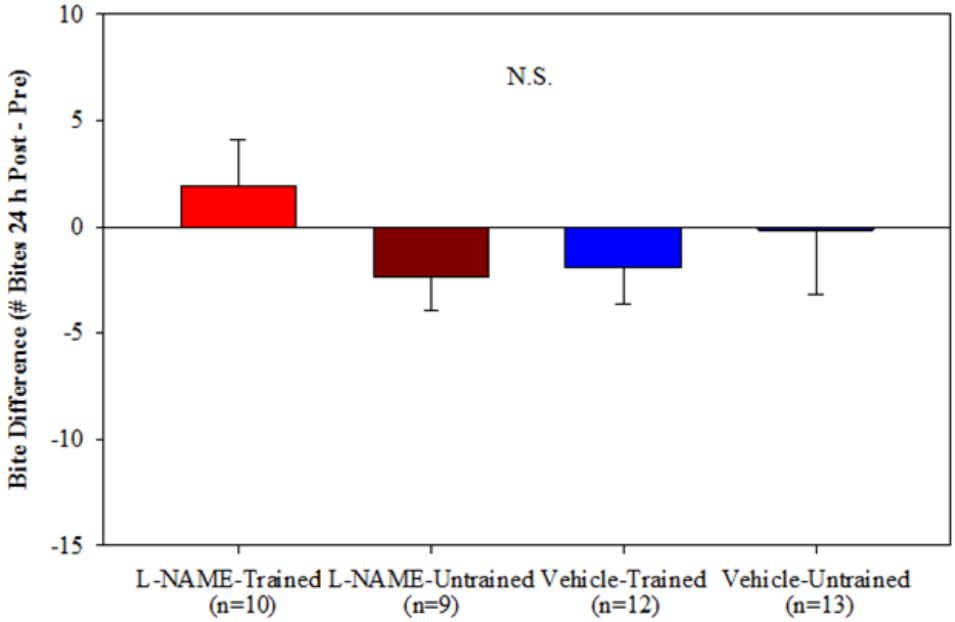

Figure 27. The effect of L-NAME on feeding behavior 24 h after single-trial training.

Synopsis of behavioral analyses

In summary, the LTS training was shown to produce LTS of the TSWR (Scholz and Byrne, 1987; Goldsmith and Byrne, 1993; Cleary et al., 1998) and the concomitant suppression of feeding behavior (Acheampong et al., 2012; Shields-Johnson et al., 2013), but both of these effects of LTS training were blocked in trained animals injected with L-NAME. The single-trial training was shown to induce suppression of feeding behavior evident at 15 min and 2 h post-training, but not at 24 h (Acheampong et al., 2012). The effect of single-trial training was also blocked in trained animals injected with L-NAME. The behaviors of untrained animals did not differ depending on treatment for either experiment. These results suggested that modifications of both defensive and non-defensive behaviors via aversive learning were blocked when NO signaling was inhibited.

From behavior to the neurophysiological correlates

After examining the effects of inhibiting NO on feeding behavior in *Aplysia*, the effects of altering NO availability on the neural circuit underlying feeding behavior were investigated. The next series of experiments focused on neuron B51, which is a decision-making cell whose excitability and activity determine feeding behavior produced *in vivo*. B51 is also a site for plasticity in feeding behavior, as it has previously been shown that the suppression of feeding behavior induced by LTS training is manifested by the decrease in B51 excitability and activity (Acheampong et al., 2012; Shields-Johnson et al., 2013). The purpose of these experiments was to examine the role

of NO signaling in modulating fictive feeding behavior (i.e., the neurophysiological correlates of feeding behavior) via alteration of B51 properties.

Effects of L-NAME in vitro *on B51 excitability*

The purpose of this experiment was to investigate the role of NO in the modulation of B51 excitability by blocking the NO signaling pathway. L-NAME treatment *in vitro* significantly decreased the BT of B51 (percent change in BT; L-NAME: -19.18±7.65, n=10; Vehicle: 10.91±5.25, n=11; $p < 0.05$, U=13.000; Fig. 28). This significant decrease in BT corresponds to a significant increase in the excitability of B51. L-NAME treatment also significantly increased the duration of the BT plateau potentials (percent change in BT plateau duration; L-NAME: 117.26±45.95, n=10; Vehicle: -12.67±8.36, n=10; $p < 0.05$, U=15.000; Fig. 29), i.e., the duration of the plateau potential elicited when the cell's BT was reached. These alterations are displayed in representative intracellular recordings of B51 during BT determination for L-NAME and vehicle pre- and post-bolus application measurements (Fig. 30).

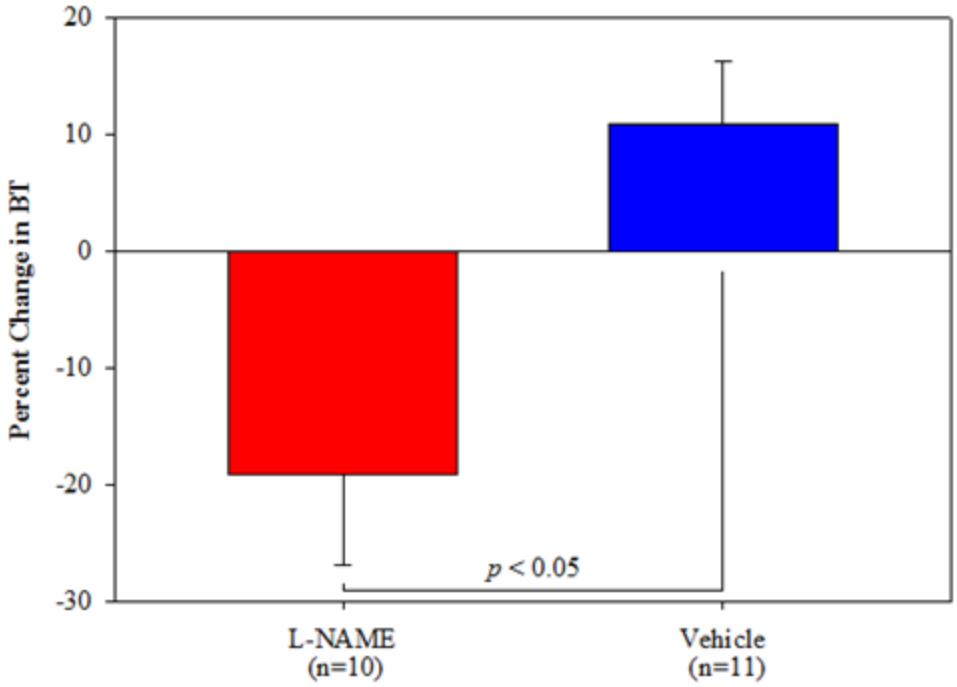

Figure 28. The effect of L-NAME *in vitro* on B51 BT.

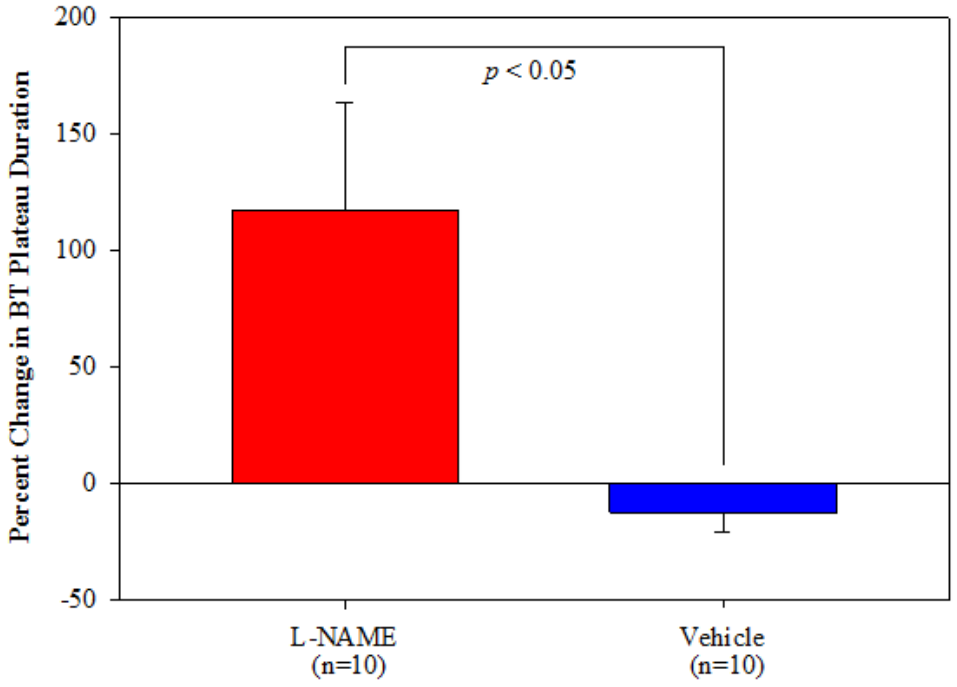

Figure 29. The effect of L-NAME *in vitro* on B51 BT plateau potential duration.

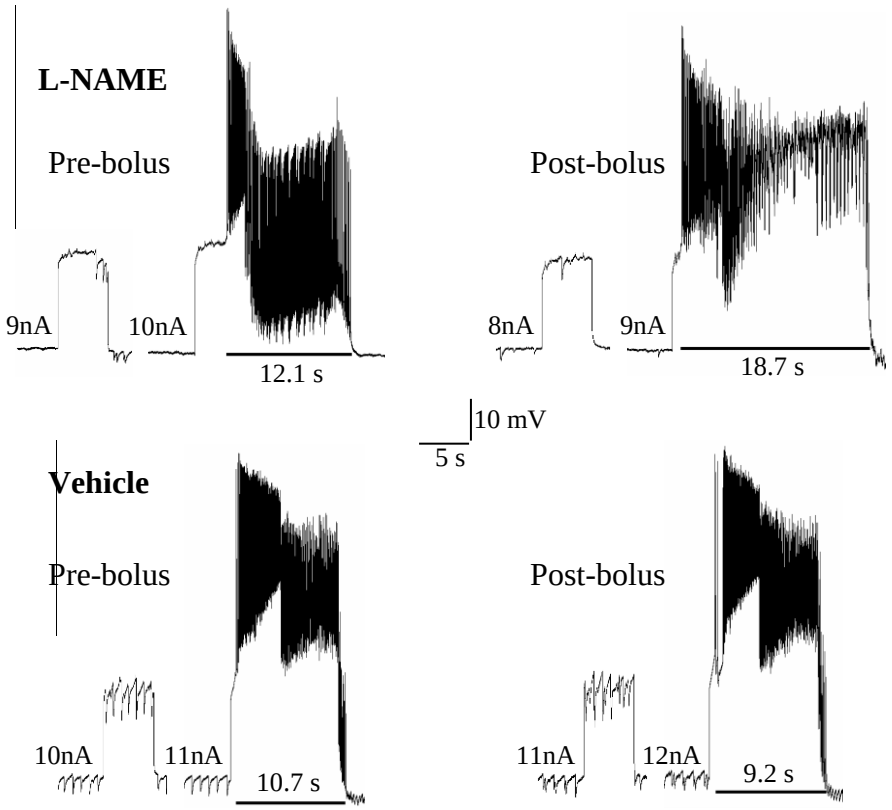

Figure 30. Representative intracellular recordings from B51 illustrating the measurement of BT and plateau potential duration for L-NAME (top) and vehicle (bottom) pre-bolus (left) and post-bolus (right).

L-NAME treatment *in vitro* produced no significant difference in B51 V_m (percent change in V_m; L-NAME: 8.21±1.54, n=10; Vehicle: 7.91±2.11, n=11; $p = 0.860$, U=52.000; Fig. 31) or R_{in} (percent change in R_{in}; L-NAME: -2.72±2.00, n=10; Vehicle: 0.00±2.00, n=11; $p = 0.307$, U=40.000; Fig. 32). These findings, when considered with the effect of L-NAME on the BT and BT plateau potential duration, demonstrated that the effect of NO on B51 is not on resting membrane properties, but rather on voltage-dependent channels.

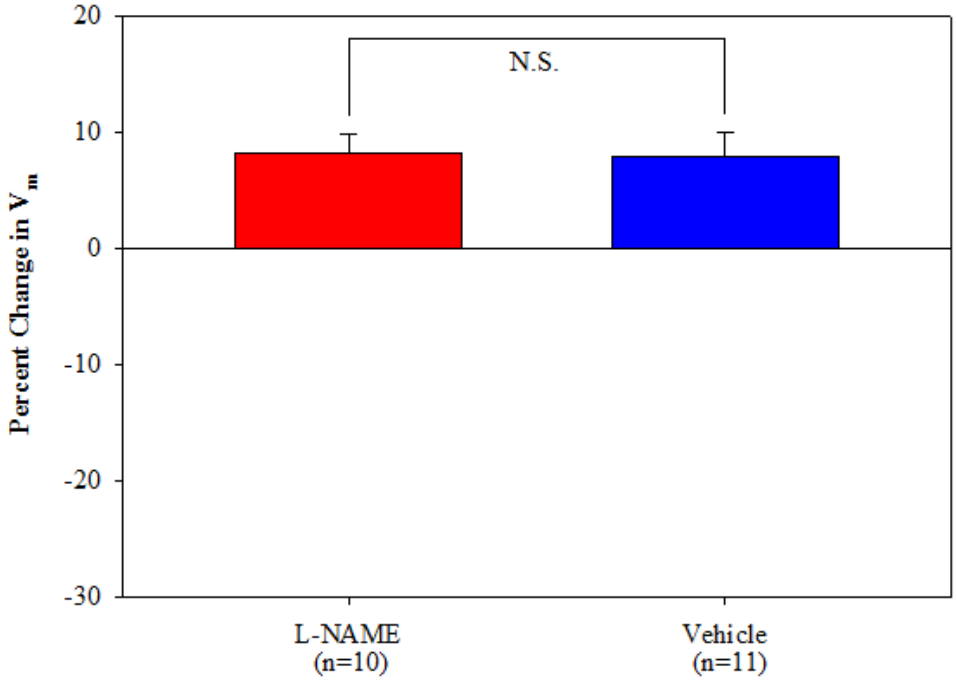

Figure 31. The effect of L-NAME *in vitro* on B51 V_m.

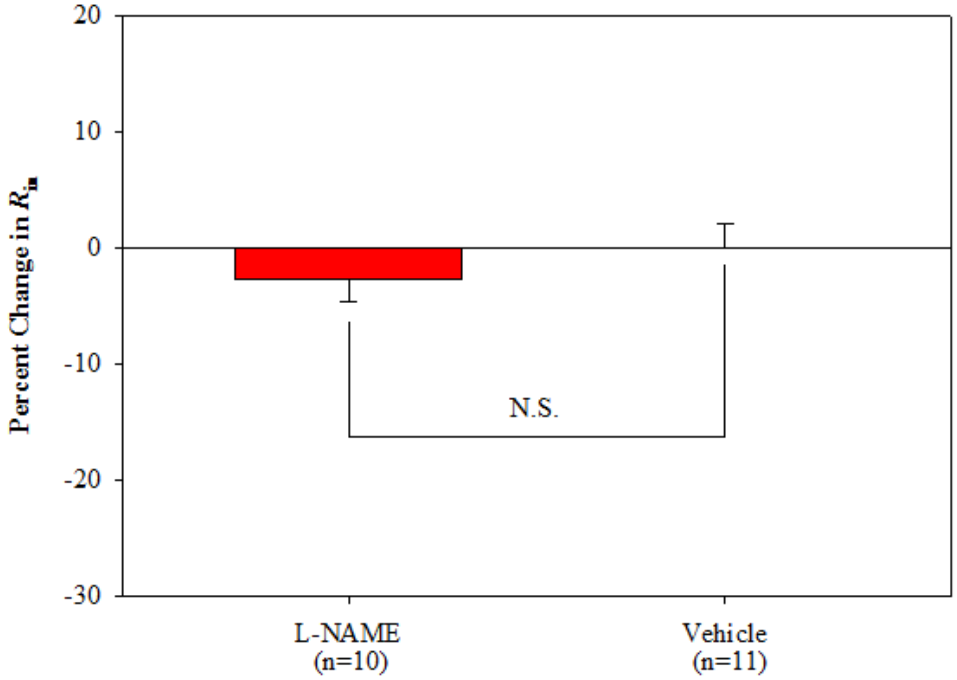

Figure 32. The effect of L-NAME *in vitro* on B51 R_{in}.

Effects of L-NAME in vitro *on B51 activity*

The purpose of this experiment was to investigate the role of NO in CPG-driven B51 activity. To drive B51 and recruit it into the CPG, this experiment used monotonic electrical stimulation of Bn.2,3 known to activate the feeding CPG and evoke fictive feeding patterns comprised of the neuronal correlates of feeding behavior (Nargeot et al., 1997, 1999a; Mozzachiodi et al., 2008). L-NAME treatment *in vitro* did not affect the latency to the first plateau potential during Bn.2,3 stimulation (latency to first stimulated plateau; L-NAME: 37.33±12.19, n=8; Vehicle: 32.70±6.80, n=8; $p = 0.959$, U=31.000; Fig. 33). Although there was no significant difference of treatment *in vitro* on the number of plateau potentials elicited during Bn.2,3 stimulation (number of stimulated plateaus; L-NAME: 2.44±0.58, n=9; Vehicle: 3.75±1.11, n=8; $p = 0.519$, U=29.000; Fig. 34), L-NAME treatment did significantly increase the duration of the stimulated plateau potentials, i.e., the plateau potentials elicited during Bn.2,3 stimulation (stimulated plateau duration; L-NAME: 12.32±1.14, n=22; Vehicle: 8.80±0.66, n=30; $p < 0.05$, U=194.000; Fig. 35).

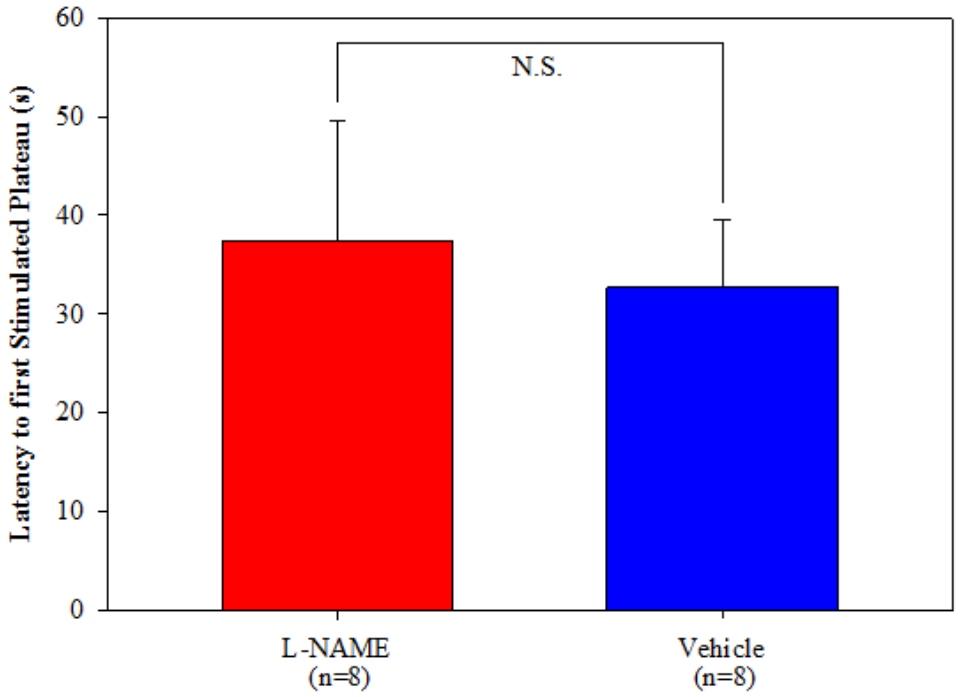

Figure 33. The effect of L-NAME *in vitro* on the latency to the first plateau potential during Bn.2,3 stimulation of B51.

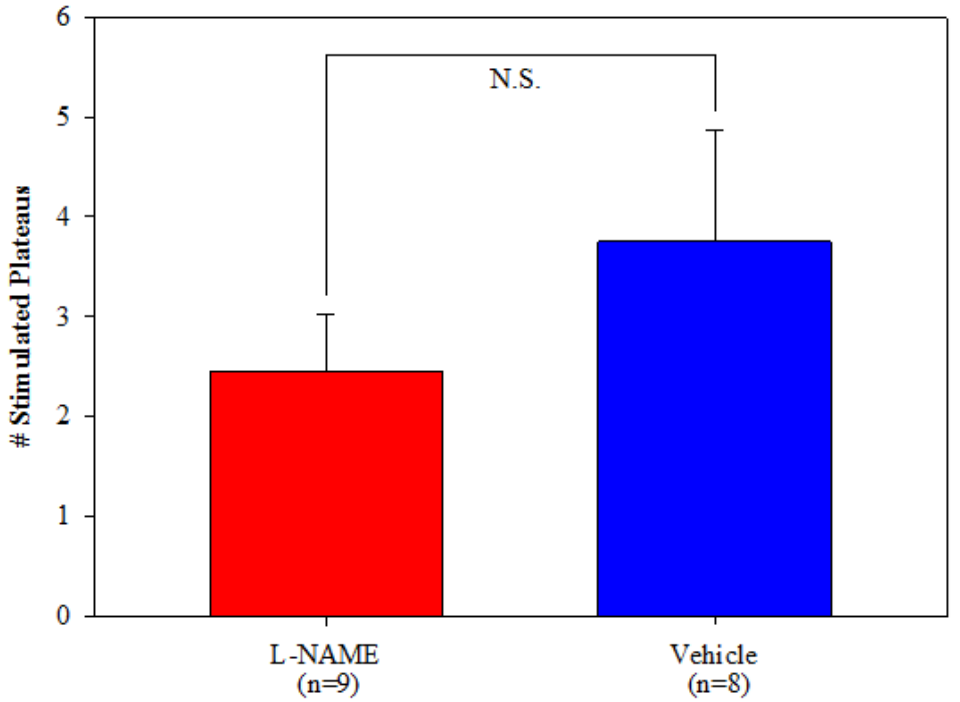

Figure 34. The effect of L-NAME *in vitro* on the number of plateau potentials elicited during Bn.2,3 stimulation of B51.

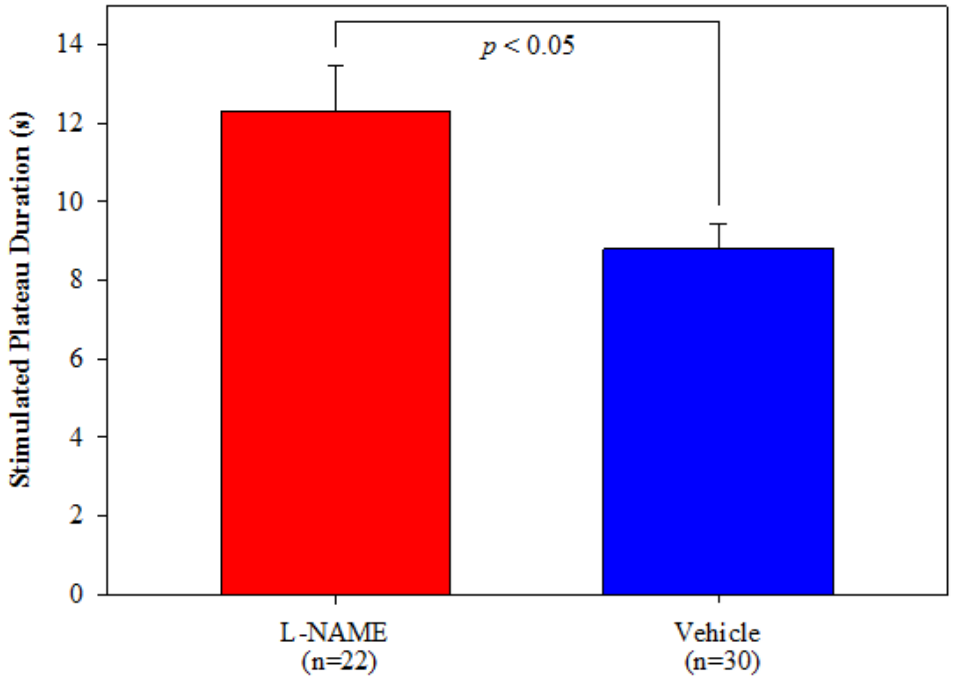

Figure 35. The effect of L-NAME *in vitro* on the duration of plateau potentials elicited during Bn.2,3 stimulation of B51. Note: for this comparison, the sample sizes represent the pooled number of plateau potentials that occurred during Bn.2,3 stimulation of B51 per treatment.

Furthermore, L-NAME treatment *in vitro* significantly decreased the occurrence of sub-threshold depolarizations elicited during Bn.2,3 stimulation (number of stimulated sub-threshold depolarizations; L-NAME: 3.78±1.28, n=9; Vehicle: 10.00±2.20, n=8; $p < 0.05$, U=14.500; Fig. 36). This finding indicated a bias in B51 away from the elicitation of eBMPs to which the sub-threshold depolarizations correlate (Nargeot et al., 1999a, b).

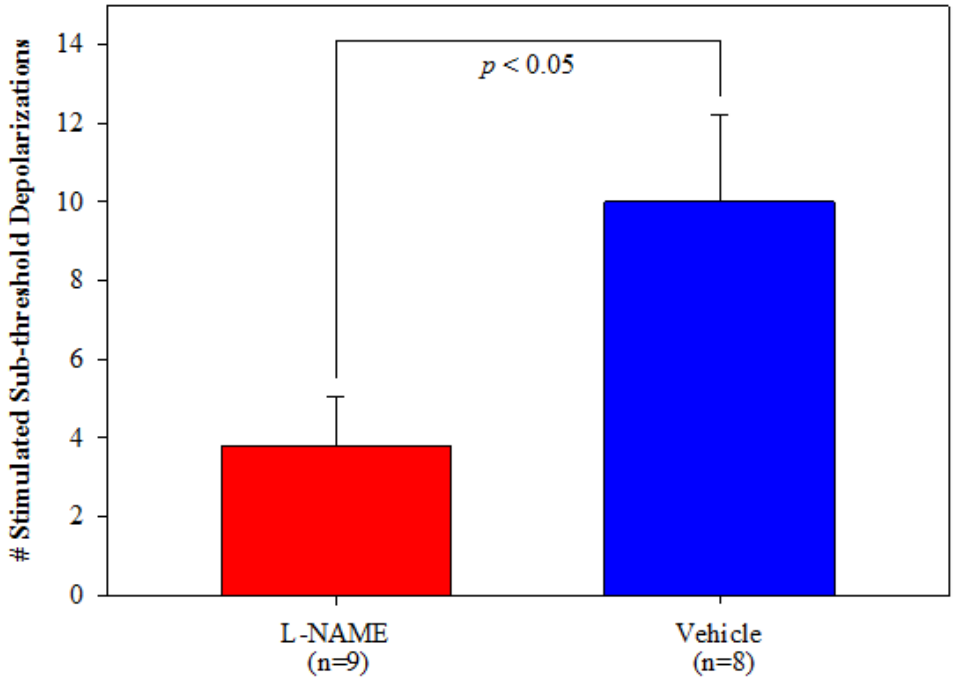

Figure 36. The effect of L-NAME *in vitro* on the number of sub-threshold depolarizations elicited during Bn.2,3 stimulation of B51.

In addition to stimulated B51 activity, L-NAME treatment *in vitro* also affected spontaneous activity. L-NAME treatment significantly increased the occurrence of spontaneous plateau potentials after the addition of the bolus (spontaneous plateau difference; L-NAME: 1.50±0.87, n=10; Vehicle: -0.60±0.31, n=10; $p < 0.05$, U=24.500; Fig. 37). However, L-NAME treatment did not affect the occurrence of spontaneous sub-threshold depolarizations (spontaneous sub-threshold depolarization difference; L-NAME: 0.10±0.50, n=10; Vehicle: -0.60±0.50, n=10; $p = 0.441$, U=40.500; Fig. 38).

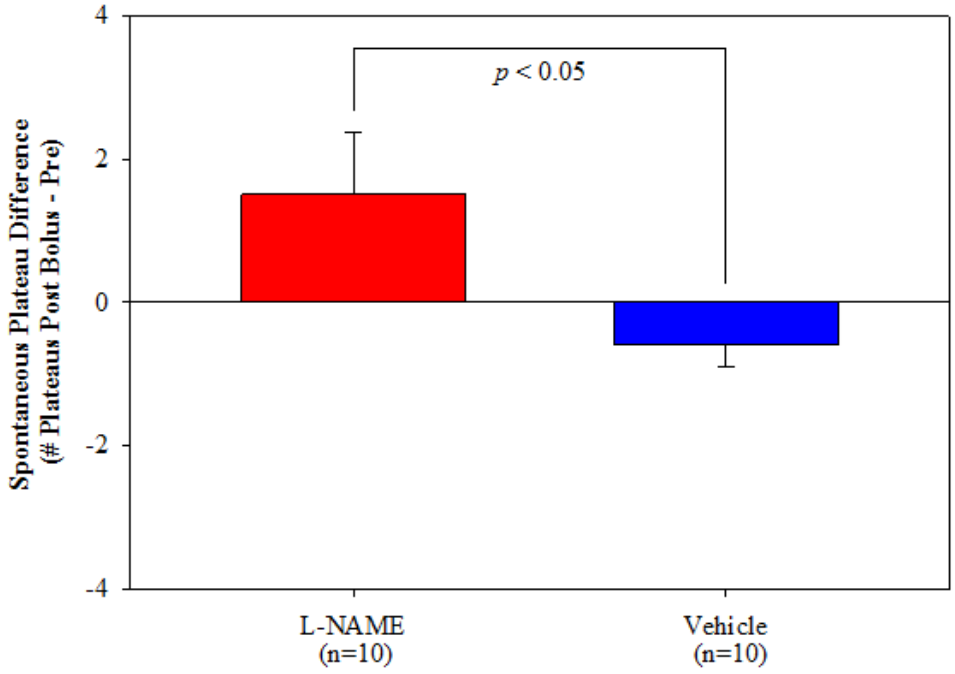

Figure 37. The effect of L-NAME *in vitro* on the number of spontaneous plateau potentials elicited pre- vs. post-L-NAME application.

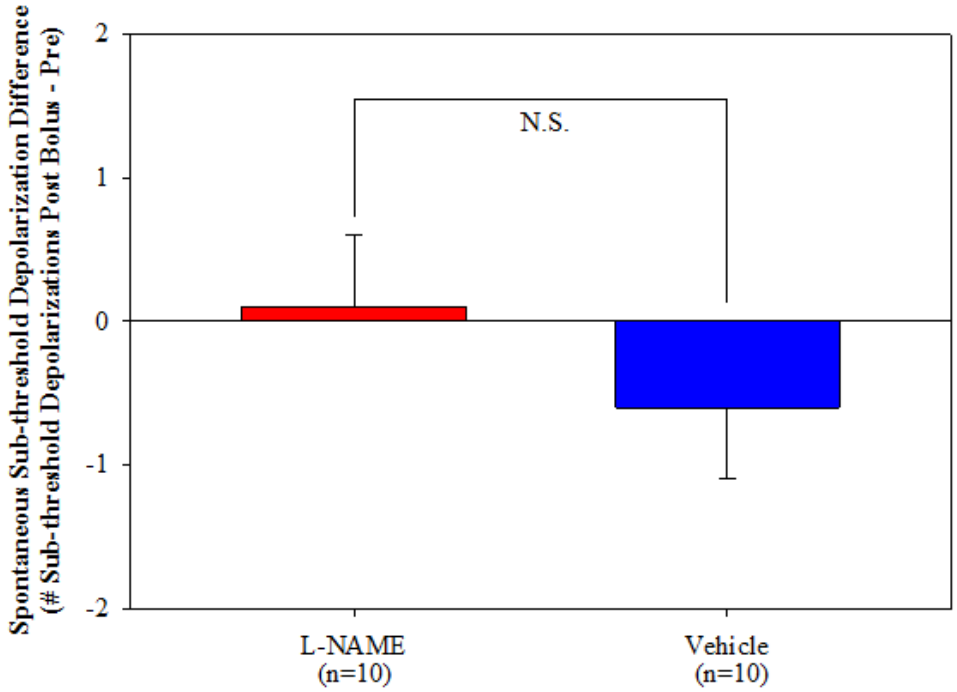

Figure 38. The effect of L-NAME *in vitro* on the number of spontaneous sub-threshold depolarizations elicited pre- vs. post-L-NAME application.

Effects of SNAP in vitro *on B51 excitability*

The purpose of this experiment was to investigate the role of NO in the modulation of B51 excitability by augmenting the NO signaling pathway via the NO donor SNAP. SNAP treatment *in vitro* significantly increased the BT of B51 (percent change in BT; SNAP: 39.87±26.41, n=5; Vehicle: -10.94±0.33, n=4; $p < 0.05$, U=0.500; Fig. 39). This significant increase in BT corresponds to a significant decrease in the excitability of B51. As seen with L-NAME treatment, SNAP treatment *in vitro* did not affect B51 V_m (percent change in V_m; SNAP: 5.86±3.10, n=7; Vehicle: 9.44±2.98, n=5; $p = 0.268$, U=10.000; Fig. 40) or R_{in} (percent change in R_{in}; SNAP: -10.42±6.59, n=7; Vehicle: -9.65±2.65, n=5; $p = 1.000$, U=17.000; Fig. 41). This experiment also demonstrated that the effect of NO on B51 is not on resting membrane properties, but rather on voltage-dependent elements, and that NO is therefore more involved in neuronal activation/inhibition.

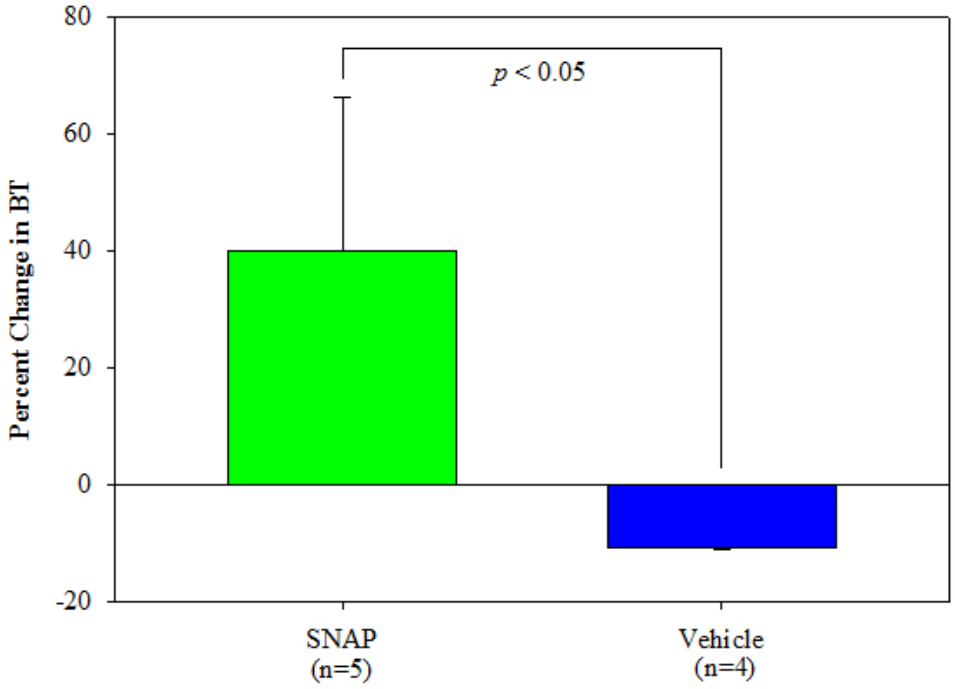

Figure 39. The effect of SNAP *in vitro* on B51 BT.

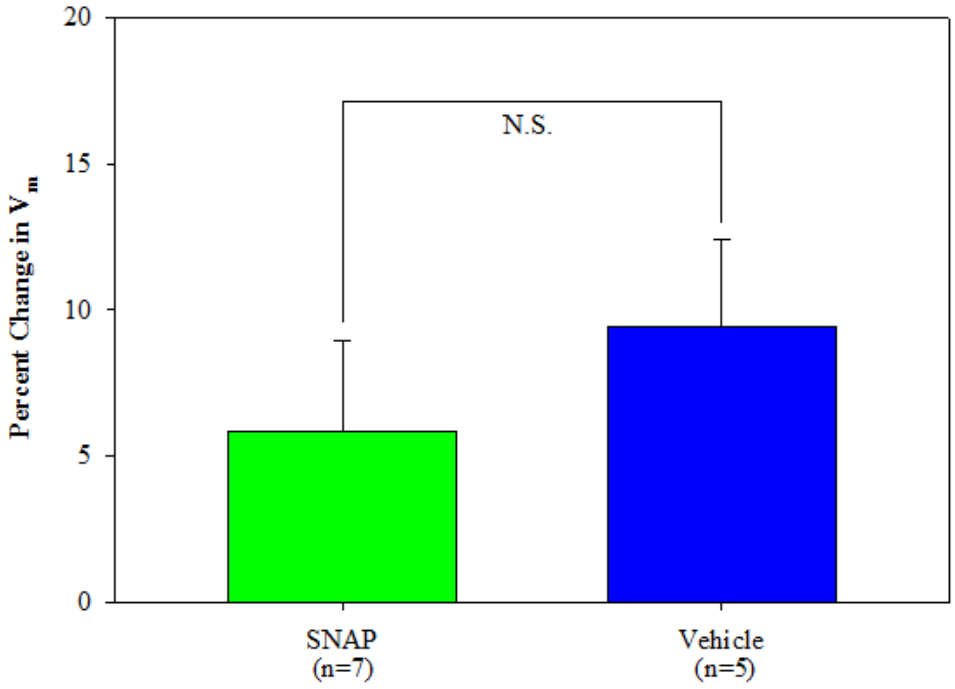

Figure 40. The effect of SNAP *in vitro* on B51 V_m.

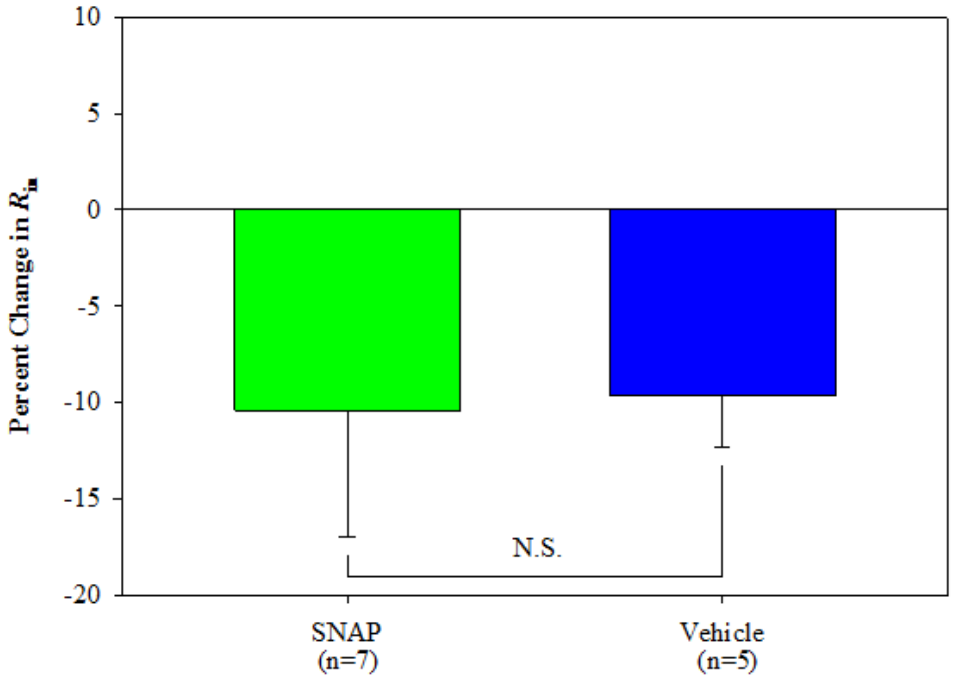

Figure 41. The effect of SNAP *in vitro* on B51 R_{in}.

Synopsis of in vitro *analyses*

In summary, L-NAME treatment, which inhibits NO synthesis, increased the excitability and activity of B51. Overall, this result corresponded to an increase in plateau potentials, an increase in the duration of plateau potentials, and a decrease in sub-threshold depolarizations. In contrast, SNAP treatment, which adds exogenous NO, decreased the excitability of B51. Together, these findings demonstrated the bidirectional modulation of B51 excitability by NO signaling.

DISCUSSION

This study yielded two primary conclusions. First, experiments with L-NAME revealed the requirement of NO signaling in the behavioral plasticity induced by exposure to sensitizing stimuli. The results manifested in three ways through the inhibition of NO synthesis: (1) L-NAME treatment suppressed the occurrence of LTS of the TSWR; (2) L-NAME treatment prevented the LTS training-induced suppression of feeding behavior; and (3) L-NAME treatment prevented the single-trial training-induced suppression of feeding behavior. The second conclusion, revealed through *in vitro* experiments with L-NAME and SNAP, is that NO exhibited bidirectional modulation of fictive feeding behavior by regulating the excitability and activity of neuron B51, a decision-making cell in the feeding neural circuit.

NO signaling is required for behavioral plasticity

L-NAME inhibits LTS of the TSWR

The training protocol used in this study is known to reliably induce LTS of the TSWR in trained animals (Scholz and Byrne, 1987; Goldsmith and Byrne, 1993; Cleary et al., 1998). This study demonstrated that L-NAME treatment prevented the occurrence of LTS of the TSWR, indicating that NO signaling is necessary for LTS to occur.

The LTS of the TSWR is mediated by 5-HT in response to sensitizing stimuli (Glanzman et al., 1989; Levenson et al., 1999; Marinesco and Carew, 2002). The LTS memory is created through a 5-HT pathway that culminates in the long-term presynaptic facilitation of the excitatory postsynaptic potential (EPSP) between the siphon sensory neurons and the siphon motor neurons controlling the TSWR (Brunelli et al., 1976;

Mackey et al., 1989; Cleary et al., 1998; Antonov et al., 2001). The increase of humoral 5-HT initiates a cyclic adenosine monophosphate (cAMP)-protein kinase A (PKA)-mitogen-activated protein kinase (MAPK)-cAMP response element-binding protein (CREB1; the *Aplysia* homologue of mammalian CREB) pathway that produces the memory (Kandel, 2001; Liu et al., 2008). The released 5-HT activates adenylyl cyclase, causing an increase in cAMP, which in turn activates PKA. The PKA translocates to the nucleus with MAPK to activate the transcription factor CREB1 in order to activate gene expression, synthesize proteins, and enhance synaptic connections that form the LTS memory of the TSWR (Brunelli et al., 1976; Martin et al., 1997; Kandel, 2001; Hawkins et al., 2006; Liu et al., 2008). The presynaptic pathways of PKA and MAPK that lead to the activation of CREB1 necessitate the use of a retrograde neurotransmitter, which is a neurotransmitter that is released postsynaptically and travels "backwards" across the synapse to bind and signal to the presynaptic neuron (Casadio et al., 1999). NO is an exemplary and common retrograde transmitter (Garthwaite and Boulton, 1995; Jacklet and Koh, 2001), and if NO functions as the retrograde messenger involved in the LTS of the TSWR, then the inhibition of NO synthesis via L-NAME treatment would be expected to prevent the LTS memory formation as seen in this study.

Furthermore, NO is directly involved in the synaptic facilitation of the EPSP between the siphon sensory neurons and the siphon motor neurons controlling siphon withdrawal under classical conditioning (Antonov et al., 2007). Treatment with L-NAME inhibited associative learning in the siphon withdrawal reflex circuit, and both the presynaptic facilitation of the sensory neurons and synaptic facilitation of the motor neurons were inhibited by treatment with a NO scavenging molecule (Antonov et al.,

2007). This inhibition makes it likely that NO directly contributes to synaptic facilitation of the EPSP of the sensorimotor neurons, and suggests the involvement of NO with presynaptic PKA activity via the 5-HT-cAMP-PKA signaling pathway (Antonov et al., 2007). It is possible that NO is an upstream neurotransmitter from 5-HT, functioning as an anterograde precursor to the 5-HT-cAMP-PKA pathway mediating the LTS of the TSWR, providing an additional potential explanation for the suppression of the LTS of the TSWR via L-NAME observed in this study.

The signaling pathway that forms the TSWR LTS memory is similar to the pathway mediating transcription-dependent sensitization of the nociceptive sensory neurons in the pleural ganglia (Lewin and Walters, 1999). An applied noxious stimulus that injures the *Aplysia* also creates a sensitization memory through the induction of long-term hyper-excitability (LTH) of the nociceptive sensory neurons. This LTH is mediated by NO via the NO-cGMP-PKG signaling pathway, which functions in parallel with the 5-HT-cAMP-PKA pathway, and it uses activated PKG and MAPK to activate transcription factors leading to gene expression and creating long-term memory (Lewin and Walters, 1999). The application of L-NAME to these nociceptive sensory neurons was shown to produce significant inhibition of the LTH, which is a correlate of memory for nociceptive sensitization. The similarity in signaling between TSWR LTS and nociceptive LTH, in addition to LTH being dependent upon NO signaling, suggests an intimate association between the two types of sensitization. This association is another possible explanation for the inhibition of LTS of the TSWR observed in this study.

L-NAME prevents the feeding suppression induced by LTS and single-trial training

LTS training has previously been shown to induce a significant suppression of feeding behavior in *Aplysia* persisting for at least 24 h in addition to concurrently induced sensitization (Acheampong et al., 2012; Shields-Johnson et al., 2013). Single-trial training has also previously been shown to induce significant feeding suppression 15 min and 2 h after training in addition to concurrently induced sensitization (Acheampong et al., 2012). In this study, the treatment of animals with L-NAME was shown to prevent the suppression of feeding behavior in animals that underwent LTS training and also in animals that underwent single-trial training.

In previous studies, L-NAME treatment was shown to prevent the formation of short-term memory in *Aplysia* that a food item was inedible (Katzoff et al., 2002; Katzoff et al., 2006), indicating that NO is involved in short-term synaptic plasticity. NO is likely involved in mediating synaptic plasticity in pre-existing synapses, possibly via modulating the release of other neurotransmitters, which corresponds to short-term memory formation (Jacklet, 1997; Kandel, 2001; Antonov et al., 2007). Therefore, it is logical that L-NAME treatment prevented the suppression of feeding at 15 min after single-trial training in this study by preventing the formation of short-term memory.

The neurotransmitter NO has additionally been linked to learning and long-term memory formation requiring gene expression and protein synthesis due to its involvement in the NO-cGMP-PKG pathway (Lewin and Walters, 1999). The inhibition of feeding behavior by tonic NO production may be due to its tonic activation of the messenger molecule guanylyl cyclase, which in turn leads to production of cGMP (Jacklet, 1997;

Miller et al., 2011a; Miller et al., 2011b; Susswein and Chiel, 2012). Both blocking guanylyl cyclase from being activated by NO and blocking NO synthesis via L-NAME treatment prevented *Aplysia* from forming long-term memory that a food item was inedible (Katzoff et al., 2006). Activation of PKG was also necessary for *Aplysia* to form long-term memory that a food item was inedible (Michel et al., 2011). These previous studies indicate that NO is essential for long-term memory. In this experiment, NO signaling pathways were inhibited by L-NAME treatment, which explains why the *Aplysia* did not express sensitization-training induced feeding suppression at 2 h following single-trial training and 24 h after LTS training.

It is important to note that the experiment presented in this thesis examined the concurrent changes occurring in two behaviors – the TSWR and feeding. While the role of NO signaling in modulating feeding behavior was somewhat expected, the aforementioned involvement and apparent necessity of NO in sensitization was an unexpected yet significant finding.

NO modulates the excitability and activity of the feeding circuit

The neurotransmitter NO is known to be involved in the feeding neural circuit of *Aplysia* (Katzoff et al., 2002). This study demonstrated that the role of NO in the feeding circuit is much more significant than merely tonic background inhibition of feeding in the absence of food stimuli (Miller et al., 2011a; Miller et al., 2011b). The treatments with L-NAME, which inhibited NO availability, and with SNAP, which increased NO availability, demonstrated the bidirectional effects of NO on B51 excitability. When NO synthesis was blocked by L-NAME, the excitability of B51 increased significantly.

Conversely, when NO concentration in the buccal ganglion was elevated by SNAP, the excitability of B51 decreased significantly. The L-NAME and SNAP treatments also indicated that the effect of NO is not on resting membrane properties (i.e., V_m and R_{in}), but rather on voltage-dependent elements.

The changes that occur in a cell's voltage-dependent elements often manifest as changes in excitability (Mozzachiodi and Byrne, 2010). The voltage-dependent excitability of B51 demonstrated in this experiment is likely based on sodium (Na^+) channels. By isolating ion channels in B51, it was previously found that the increase in B51 BT, which correlates to the LTS training-induced suppression of feeding (Acheampong et al., 2012; Shields-Johnson et al., 2013), may be mediated through a decrease in response of voltage-gated Na^+ channels to depolarizing current (Hernandez, 2012; Hernandez et al., 2013). Na^+ channels that require more input to open directly correspond to an increase in the BT of B51 and therefore a decrease in excitability. NO may act through the NO-cGMP-PKG pathway to modify the Na^+ channels via phosphorylation, increasing their resistance to triggering depolarization (Hernandez, 2012; Hernandez et al., 2013). It is possible that the tonic inhibition of feeding behavior that NO exerts operates through this ion channel modification. Therefore, blocking NO with L-NAME treatment may make lower the input required for B51 Na^+ channels to trigger a depolarization, eliciting the plateau potential at a lower BT, which coincides with the findings of this study. L-NAME might additionally keep Na^+ channels open longer, prolonging depolarization, which coincides with the observed increase in duration of plateau potentials generated by B51 treated with L-NAME.

The voltage-dependent excitability may also be based on the opening and closing of potassium (K^+) channels. The metacerebral cell (MCC) is involved in the activation of the feeding circuit in response to arousal by food stimuli (Jing et al., 2008; Katzoff et al., 2010; Miller et al., 2011b) and is indirectly inhibited at rest by the tonic production of NO, but directly excited by NO in the presence of food (Miller et al., 2011b). An increase in NO – whether by nitric oxide synthase in cerebral neuron C2 or treatment with NO donor – activates guanylyl cyclase, which produces cGMP and in turn causes the closure of background K^+ channels, depolarizing the neuron and increasing its excitability (Koh and Jacklet, 1999; Jacklet and Koh, 2001). The application of histamine, a co-transmitter of NO, to the MCC also results in its elicitation of a synaptic potential and increase in excitability mediated by the closure of K^+ channels (Weiss et al., 1986). If NO functions as an excitatory (directly) neurotransmitter for the MCC by closing K^+ channels, then it may be the case that, in the feeding neural circuit where NO is inhibitory, the inhibitory effect of NO is mediated through the opening of K^+ channels in buccal neurons that are involved in recruiting B51 into the CPG. This possibility appears to coincide with the observed increase in duration of depolarizations (i.e., plateau potentials) of B51 treated with L-NAME, as inhibiting NO would then close K^+ channels, delaying repolarization and prolonging depolarization of neurons in the feeding CPG. Also, if not acting directly on B51 K^+ channels, there would be no change in B51 resting membrane properties.

The L-NAME-induced increase in B51 excitability observed in this study also represents a shift in buccal motor pattern output of the feeding CPG. When the CPG is activated, B51 decides the type of BMP elicited, and this decision is influenced by the excitability of B51. More excitable B51s evoke more iBMPs and fewer eBMPs, and less

excitable B51s evoke fewer iBMPs and more eBMPs (Nargeot et al., 1999a, b; Kabotyanski et al., 2000). L-NAME treatment yielded fewer sub-threshold depolarizations during Bn.2,3 stimulation. The decrease in sub-threshold depolarizations, which are the neural correlates of eBMPs, increased the likelihood of occurrence of plateau potentials, i.e., the neural correlates of iBMPs (Nargeot et al., 1999a, b; Kabotyanski et al., 2000). This reduction of inhibition of iBMP generation is evidenced by the increase in occurrence of spontaneous plateau potentials following L-NAME treatment observed in this study, which also coincides with the increased excitability of B51 as the excitability of this decision-making neuron determines the type BMP elicited (Kabotyanski et al., 2000).

In *Aplysia*, this shift in the output of the feeding CPG, with higher excitability producing more iBMPs, is largely mediated by dopamine (DA) (Kabotyanski et al., 2000). DA is not involved in the effects of sensitization training, but it is significantly involved in pattern generation in the CPG as well as the increase of feeding behavior induced by operant conditioning (Nargeot et al., 1999c; Brembs et al., 2002; Reyes et al., 2005). Buccal neurons B31/32 are part of the feeding CPG involved in the rhythmic production of iBMPs (Moroz, 2006; Hurwitz et al., 2008). Sustained depolarization of B31/32 initiates radula protraction, which is the first stage of consummatory feeding, pending the decision of B51 (Hurwitz et al., 2008). NO was found to tonically inhibit B31/32 depolarization via tonic activation of guanylyl cyclase and tonic production of cGMP, which closes the leak current that when opened enables depolarization of the coupled cells (Miller et al., 2011a; Susswein and Chiel, 2012). DA can overcome the low-level inhibition and depolarize B31/32, activating the feeding CPG in a manner that

it elicits more iBMPs and fewer eBMPs (Nargeot et al., 1999c; Kabotyanski et al., 2000).

Increased concentrations of NO, such as from a NO donor, were found to inhibit the effects of DA via guanylyl cyclase activation in abdominal neurons in *Aplysia kurodai* (Sawada et al., 1997). Additionally, as was observed in this study, the NO-mediated action did not affect the resting membrane properties of the target cells (Sawada et al., 1997). Although DA is an important neurotransmitter, NO signaling pathways appear to be upstream from and modulatory of DA (Sawada et al., 1997).

Conclusions and Future Directions

Although a structurally-simple signaling molecule, NO was found to have significant effects on the plasticity of multiple *Aplysia* behaviors and underlying neural circuits. The treatment of L-NAME *in vivo* was found to prevent the occurrence of LTS of the TSWR in trained animals. L-NAME treatment *in vivo* was also found to prevent both the LTS and single-trial training-induced suppression of feeding behavior. The treatment of L-NAME *in vitro* significantly increased the excitability and activity of the feeding neural circuit via modulation of the decision-making neuron B51, shifting the CPG toward the production of more ingestive patterns. In contrast, SNAP treatment *in vitro*, which increased NO, significantly decreased the excitability of B51. It is possible that sensitization training in *Aplysia* leads to an upregulation of nitric oxide synthase activity and thus a concurrent increase in NO. When this possibility is considered in the context of the results of this experiment, it may provide the underlying mechanism for and bridge between the concomitant enhancement of the TSWR and suppression of feeding behavior induced by sensitization training (Acheampong et al., 2012; Shields-Johnson et al., 2013).

The first step that should be taken for future experiments is the *in vitro* analysis of B51 properties and activity that are the correlates of the *in vivo* behavioral data (i.e., TSWR and feeding) to determine if the same trends are visible. In the B51 neurons examined following the animal's completion of the behavioral post-tests, the concomitant enhancement of the TSWR and suppression of feeding behavior via LTS training typically manifests as a high B51 BT, which is indicative of decreased excitability (Shields-Johnson et al., 2013). It would be notable and expected if L-NAME treatment similarly prevented the effects of LTS training in *in vitro* behavioral correlates.

Future studies should also investigate the *in vivo* effects of treatment with NO donor molecules. If inhibiting NO synthesis via L-NAME blocked the enhancement of the TSWR and suppression of feeding typically elicited by LTS training (Acheampong et al., 2012; Shields-Johnson et al., 2013), then enhancing NO concentrations may augment those outcomes. Additionally, SNAP was found to be able to induce memory formation at previously sub-threshold stimuli (Katzoff et al., 2006). It would be interesting if the amplification of neurotransmitter effects by SNAP and the increase in NO could induce LTS training effects from single-trial training.

It is also important to determine at what point along the NO signaling pathways the signals diverge to affect different behaviors in *Aplysia*. This can be accomplished by inhibiting signaling molecules downstream from NO and assessing the change or lack of change in the animals' behaviors. Treatments should include methylene blue to inhibit guanylyl cyclase signaling and cGMP production (Katzoff et al., 2006), KT5823 to inhibit PKG signaling (Matsumoto et al., 2006), SQ22536 to inhibit adenylyl cyclase signaling and cAMP production (Matsumoto et al., 2006), KT5720 to inhibit PKA

signaling (Matsumoto et al., 2006), and anti-apMAPK antibodies or PD98059 to inhibit MAPK activation (Martin et al., 1997; Michel et al., 2011). Training protocols should include both single-trial and LTS training, and experiments should examine the TSWR and feeding behavior (Acheampong et al., 2012). A similar study should also be conducted that involves the injection of L-NAME followed by 5-HT treatment, which is known to induce LTS but not feeding suppression (Levenson et al., 1999; Shields-Johnson et al., 2013). The outcomes of that study would yield more insight into the position of NO in the 5-HT signaling pathway.

The neurotransmitter NO appears to function at sites upstream from many signaling pathways and is clearly a highly significant signaling molecule involved in both short- and long-term synaptic facilitation and modulation of multiple behaviors in *Aplysia*. However, the impacts of the necessity of NO signaling in both behavioral and cellular plasticity transcend *Aplysia*, as NO and its various pathways are nearly ubiquitous across phyla regardless of the intricacy of the nervous system. Continued investigation of NO signaling in a simpler, model organism such as *Aplysia* will create new perspectives and offer unprecedented insights about the modulation of behavioral and cellular plasticity in more complex nervous systems.

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