Introduction

The origins of neuroscience

In present day, it is common knowledge that the brain, the spinal cord and all of the nerves in our body are responsible for sensing our environment, computing sensory information, and executing decisions to fit environmental changes. Despite the fact that the brain has clear connections with all organs and tissues in the body, its current understanding was not readily appreciated throughout history. It is true that trepanation, a procedure in which holes are made in the skull for healing purposes, has been observed in 7,000 year old human skulls (Alt et al., 1997); but there is insufficient evidence supporting the logic behind the treatments. However, history does provide evidence (approximately 384-322 B.C.; Bear et al., 2006) that influential scholars, such as Aristotle, were convinced that the heart, not the brain, was the center of intellect.

It was during the fourth century B.C. that keen observations by scholars and careful dissections of the brain by physicians (Bear et al., 2006) brought the natural sciences closer to understanding the true role of the central nervous system (CNS). Over the next millennium, research (e.g., Schwann and Schleyden, 1847; Ramón y Cajal, 1894) made possible the discovery of neurons, the key biotic constituents of the nervous system in Eumetazoans (i.e., all animals except for sponges, placozoans and mesozoans; Margulis and Schwartz, 1988). If it were not for neurons, it would be difficult for any organism to produce not only behavior, but two of the most successful, and complex, physiological adaptations in the animal kingdom: learning and memory.

Learning and memory

Animals encounter challenging obstacles in their environment and have the ability to modify their behavior from their experiences (i.e., learning; Byrne et al., 2009), thus increasing the possibility of survival (Kandel, 2001; Bear et al., 2006; Fioravante et al., 2008). In order to better understand behavior, scientists began to investigate animals' ability to learn in a laboratory setting (e.g., Thorndike, 1898; Pavlov, 1927). For example, the well-known Skinner Box apparatus is used to train an animal (traditionally a rat) to associate pressing a lever with food being dispensed, thus enhancing the lever-pressing behavior (Hayes, 2000; Chance, 2003). Conversely, if the lever delivers a shock when touched, the rat is less likely to press the lever (i.e., behavior is suppressed; Skinner, 1938; Chance, 2003). These experience-induced changes in behavior are examples of learning (Fioravante et al., 2008) and the persistence of behavioral changes for extended periods of time are a product of memory (Fioravante et al., 2008). It is important to note that without learning and memory, the nervous system would be still capable of producing complex functions, but would be unable to appropriately modify behavior in response to the environment (Okano et al., 2000). Therefore, learning and memory are two well-studied fields in neuroscience.

Associative and nonassociative learning

Traditionally, learning is categorized into two forms: associative and nonassociative (Bear et al., 2006). Associative learning occurs when an animal learns the relationship between stimuli that are closely associated with each other, and is categorized into two main forms: classical and operant conditioning. Classical conditioning involves the association between an unconditioned stimulus (e.g., meat that

elicits a salivation response in a carnivore) and a conditioned stimulus (e.g., a bell that precedes the meat, acting as a predictor). After sufficient training, the conditioned stimulus is able to evoke the same response as the unconditioned stimulus (Mozzachiodi and Byrne, 2010). Operant conditioning occurs when an animal learns that one of its behaviors (e.g., a rat deciding to move to a different side of a cage) has a certain consequence (e.g., the animal is not shocked), resulting in modifications of future decision making contingent on behavioral consequences (Skinner, 1938; Nargeot and Simmers, 2011).

Unlike associative learning, nonassociative learning represents an organism's response to a stimulus (or many stimuli) that occurs without a relationship to another stimulus. Nonassociative learning can be categorized into different forms, including: habituation and sensitization. Habituation is a gradual waning of a behavioral response to a weak or moderate stimulus presented repeatedly (Thompson and Spencer, 1966; Fioravante et al., 2008 Byrne et al., 2009), whereas sensitization is the enhancement of a behavioral response to a weak stimulus by presentation of a noxious stimulus (Kandel, 2001; Bear et al., 2006; Hawkins et al., 2006; Fioravante et al., 2008).

The above four traditional associative and nonassociative learning paradigms are ubiquitous in invertebrates and vertebrates (Bear et al., 2006). However, in the CNS of mammals, the subtle cellular and molecular changes that underlie learning and memory often prove difficult to define with such small neurons that participate in complex circuitry (Kandel, 2001; Hawkins et al., 2006). In order to better understand the fundamental neural mechanisms underlying the above four learning paradigms, scientists turned to the more tractable nervous systems of invertebrates (e.g., Carew, 2000; Roberts

and Glanzman, 2003; Hawkins et al., 2006; Castellucci, 2008; Byrne et al., 2009; Byrne and Roberts, 2009; Mozzachiodi and Byrne, 2010).

Using a tractable model system for studying learning and memory

When analyzing changes in behavioral output induced by learning, it is important to study model organisms that display a range of behaviors which can be readily evoked and quantified in laboratory settings (Krogh, 1929; Kupfermann, 1974). Also, when trying to investigate the cellular basis of learning-induced changes in behavior, it is desirable to study model organisms whose behaviors rely on well-characterized neural circuits amenable for single-cell analysis. These are characteristics of the marine mollusk *Aplysia californica*, which allow scientists the crucial advantage of relating particular physiological changes in the nervous system to the production of behavior (Cleary et al., 1995; Evans and Cropper, 1998; Cropper et al., 2004; Baxter and Byrne, 2006; Castellucci, 2008). For these reasons, *Aplysia* has been extensively studied (Kandel, 2001).

Using the tail-siphon withdrawal reflex to study sensitization in *Aplysia californica*

Aplysia displays several defensive responses. For example, applying a tactile or electrical stimulus to the siphon results in the withdrawal of the siphon and gill (i.e., the siphon-gill withdrawal reflex; Byrne et al., 2009). Similarly, the application of a mild stimulus to the tail initiates a withdrawal of the tail and the siphon (i.e., the tail-siphon withdrawal reflex; TSWR; Byrne et al., 2009). These behaviors allow for quantitative analysis. For example, the duration of the TSWR, from the initiation of the contraction to the onset of relaxation of the siphon, is commonly used as a measure of reflex strength (e.g., Goldsmith and Byrne, 1993; Cleary et al., 1998; Wainwright et al., 2002). Much

research on *Aplysia* has focused on sensitization, which manifests as the enhancement of the withdrawal reflexes (Frost et al., 1985; Goldsmith and Byrne, 1993; Cleary et al., 1995; Kandel, 2001; Hawkins et al., 2006).

Sensitization, like most forms of learning, can last for an extended period of time depending on the training paradigm (Cleary et al., 1995; Wainwright et al., 2002; Fioravante et al., 2006; Byrne et al., 2009). When repeated noxious stimuli are applied, a persistent form of sensitization occurs, in which behavioral changes last several days to weeks (long-term sensitization, LTS; Pinsker et al., 1973; Cleary et al., 1998; Wainwright et al., 2002). In *Aplysia*, LTS of the TSWR has been examined at the behavioral, cellular and molecular levels (Kandel, 2001). Characterizing all of the major contributors to the memory of LTS in *Aplysia* is important to establish the mechanisms utilized by an organism to protect itself when in danger. In order to better understand the overall behavioral repercussions of remembering the properties of repeated noxious stimuli, a recent experiment (Acheampong et al., 2012) began exploring the effects of LTS training on behaviors of a non-defensive nature (i.e., feeding).

Feeding behavior of *Aplysia*

Feeding behavior of *Aplysia* has been studied extensively (Kupfermann and Pinsker, 1968; Kupfermann, 1974; Elliot and Susswein, 2002; Brembs et al., 2002; Cropper et al., 2004; Baxter and Byrne, 2006; Nargeot and Simmers, 2011). Feeding behavior consists of initial appetitive movements (i.e., directing head towards food source, seaweed; Kupfermann, 1974), which are followed by a consummatory phase (i.e., biting; Croll and Davis, 1981). In order to produce a bite and pull food into the buccal cavity, where seaweed is transported to the esophagus, *Aplysia* use a semi-hardened tissue

covered with rows of chitinous teeth (i.e., radula; Cropper et al., 2004). In order for *Aplysia* to grasp a piece of seaweed with its lips, two distinct movements of the radula occur: protraction and retraction. Protraction consists of the opening of the mouth and the extension of the opened radula towards the food. Following protraction, the radula retracts, closed and with seaweed, back into the buccal cavity (Kupfermann, 1974). Because the bite occurs in an all-or-nothing fashion, biting behavior is well suited for behavioral analysis (Lechner et al., 2000; Lorenzetti, 2006; Acheampong et al., 2012).

Early analysis of feeding behavior in *Aplysia* has mostly focused on the dissociation of the appetitive and biting phases (Kupfermann, 1974) and the neural basis of biting (Elliot and Susswein, 2002; Cropper et al., 2004). Also, previous research has focused on the cellular basis of associative learning-induced changes in feeding behavior (Lechner et al., 2000; Brembs et al., 2002; Elliott and Susswein, 2002; Baxter and Byrne, 2006; Lorenzetti et al., 2006). However, there are scarce experiments investigating the effects of noxious stimuli on feeding behavior in *Aplysia* (Kupfermann and Pinsker, 1968; Acheampong et al., 2012). Work done by Acheampong et al. (2012) investigated the effects of noxious stimuli on feeding behavior in *Aplysia* using a previously-established LTS training protocol (Cleary et al., 1998; Khabour et al., 2004).These authors found that the LTS of the TSWR in *Aplysia*, seen 24 h after training, is accompanied by a suppression of biting behavior (Acheampong et al., 2012). Recent work also provides evidence that when the memory for LTS is no longer present (i.e., 72 h post training); the animal no longer expresses a suppression of biting behavior (Hernandez et al., 2011). These experiments provide evidence that the two behaviors can be temporally modified by LTS training concomitantly (Hernandez et al., 2011;

Acheampong et al., 2012). Given the well-characterized nervous system of *Aplsyia,* as well as the importance of biting behavior to the survival of the organism, work has begun characterizing the effects of LTS training on the part of the nervous system controlling biting behavior (Shields-Johnson et al., 2009; Hernandez et al., 2011).

The neural circuitry controlling biting behavior in *Aplysia*

In *Aplysia*, motor activity underlying biting behavior is produced by a central pattern generator (CPG) primarily located in the buccal ganglion (Nargeot et al., 1997; Elliott and Susswein, 2002; Cropper et al., 2004; Nargeot et al., 2009). CPGs are neuronal circuits that, when activated, are able to produce rhythmic motor patterns underlying several behaviors in both vertebrates and invertebrates (e.g., walking, breathing, flying and swimming; Grillner, 1981; Pearson, 2000; Marder and Bucher, 2001). The buccal ganglion is of particular interest because it contains all of the motor neurons and interneurons for the feeding CPG that control feeding musculature of the radula (Kupfermann, 1974; Fiore and Geppetti, 1981; Scott et al., 1991). It is also important to note that *in vitro* preparations of an isolated buccal ganglion retains the ability to generate activity associated with biting behavior (Nargeot et al., 1999a,b; Cropper et al., 2004; Baxter and Byrne, 2006).

The feeding CPG is capable of generating two distinct buccal motor patterns (BMPs): ingestion (intake of food), egestion (rejection of food) (Nargeot et al., 1999a,b). In order to categorize a BMP in an isolated buccal ganglion, recordings were made simultaneously from several nerves which innervate the radula musculature: In 2, R n.1 and n.2,1 (Nargeot et al., 1997; Nargeot et al., 1999a,b). BMPs begin with bursting activity in I2 n (i.e., protraction activity in a BMP; Hurwitz et al., 1996) and ended with

bursting activity of n.2,1 (i.e., retraction activity in a BMP; Nargeot et al., 1997). R n.1 activity (i.e., activity from radula closure motor neurons, including neuron B8; Morton and Chiel 1993b; Nargeot et al., 1997) plays a critical role in differentiating ingestion and egestion patterns. BMPs are classified as ingestion if there is a greater than 50% R n.1 activity occurring after protraction activity from I2 n is finished, whereas they are classified as egestion if R n.1 activity occurred only during protraction activity (Nargeot et al., 1999a,b). The experiments conducted by Nargeot and colleagues in 1999, which validate previous findings by Morton and Chiel in 1993, provided strong evidence that ingestion patterns produced by the feeding CPG in the buccal ganglia underlies biting behavior in *Aplysia*. Furthermore, a strong correlation was drawn between ingestion patterns and the activity of a key neuron in the feeding CPG, neuron B51 (Plummer and Kirk, 1990; Nargeot et al., 1999a,b; Baxter and Byrne, 2006; Nargeot and Simmers, 2011). Within an ingestion pattern, B51 activity occurs during n. 2,1 activity (Nargeot et al., 1999a,b), eliciting closure of the radula alongside retraction. The role of B51 in producing ingestion BMPs can also be seen with artificial manipulations of B51 activity. Depolarization-induced B51 plateau potentials make an ongoing pattern an ingestion pattern (Nargeot et al., 1999b). Conversely, if B51 is hyperpolarized (i.e., silenced) during an ongoing pattern, motor pattern activities from the feeding CPG are not ingestion patterns (Nargeot et al., 1999b). It is important to note that B51's plateau potential is intrinsic, meaning B51 maintains the ability to plateau when isolated in culture (Brembs et al., 2002; Lorenzetti et al., 2006; Mozzachiodi et al., 2008; Lorenzetti et al., 2011). Because the occurrence of ingestion activity is contingent upon neuron

B51's activity, B51 is considered a decision-making neuron (Nargeot et al., 1999a,b; Lorenzetti et al., 2008; Mozzachiodi et al., 2008; Nargeot and Simmers, 2011).

The role of B51 in the suppression of biting behavior, 24 h after LTS training

It has been shown that 24 h after LTS training, the suppression of biting behavior was accompanied by a decrease in excitability of neuron B51 (Shields-Johnson et al., 2009), manifested as an increase in burst threshold (defined as the minimum amount of depolarizing current necessary to elicit a plateau potential; *Fig. 1A*; Shields-Johnson et al., 2009). Given that biting behavior is suppressed 24 h after LTS training, a decrease in its excitability is consistent with the suppression of biting behavior observed following LTS training.

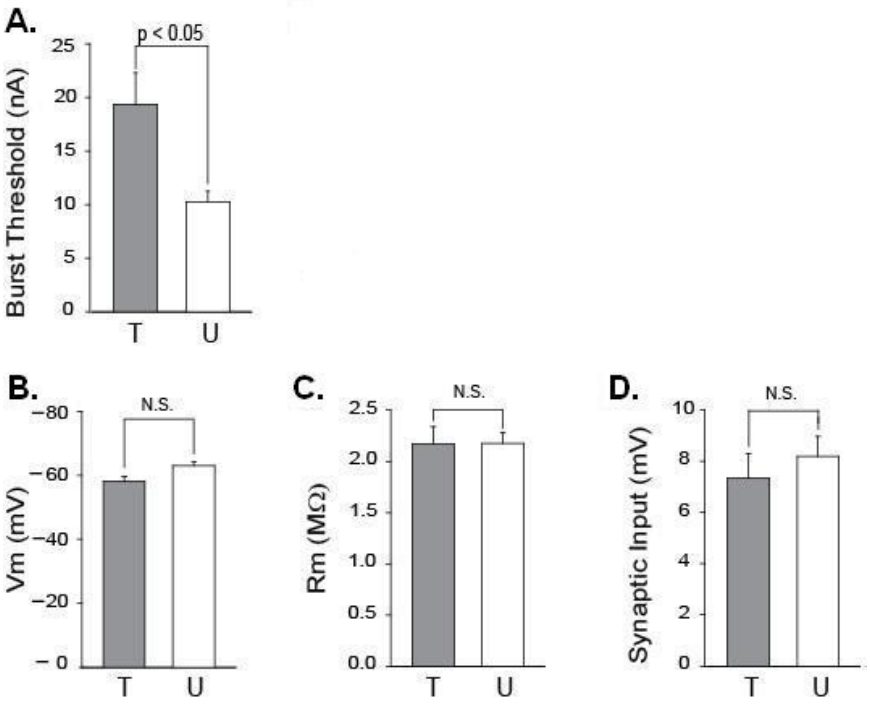

Figure 1. Effects of LTS training on B51 membrane properties. LTS training significantly increased the burst threshold (A). LTS training had no effect on the resting membrane potential (Vm, B), input resistance (Rm, C), or synaptic input (D) of B51. All of these measurements were taken 24h after treatment and the data represents the mean ± SEM, with a significance set at $p < 0.05$. Trained (T) and untrained (U) groups were compared using the Mann-Whitney test (Shields-Johnson et al., 2009).

Excitability is primarily determined by resting and voltage-dependent conductances (Mozzachiodi and Byrne, 2010), but LTS training did not induce any changes in B51 resting properties, such as resting membrane potential (Vm) or input resistance (Rm) (*Fig. 1B, C;* Shields-Johnson et al., 2009). Vm describes the ion permeability at rest, and Rm is a measure of injected currents' movement through the plasma membrane, providing an index for the relative number of channels open at rest (e.g., leaky or non-voltage dependent ion channels; Bear et al., 2006). Changes in the synaptic input to neuron B51 were also not observed following LTS training (*Fig. 1D*; Shields-Johnson et al., 2009). Because no change in resting properties or synaptic input were observed, modulation of voltage-dependent ion currents seems to account for the decrease in B51 excitability seen at 24h after LTS training.

Voltage-dependent ion channels: the sentries regulating neuronal excitability

Pioneer work analyzing changes in neuronal currents of intact axons of the squid *Loligo*, using the voltage clamp technique (Hodgkin and Huxley, 1952a,b), paved the way for the characterization of voltage-dependent conductance's' role in neuronal activity. Voltage-dependent ion channels generate electric signals, and are ubiquitously found in species from bacteria to humans (Miller, 2009; Catterall, 2010). More importantly, the mechanisms by which these channels function are conserved through evolution (Catterall, 1984; Rudy, 1988; Levitan and Kaczmarek, 1997; Jiang et al., 2002b; Bear et al., 2006); voltage-dependent ion channels act, as their name suggests, with changes in voltage. It is this unique voltage-dependent feature which plays a significant role in a neurons' excitability (Mozzachiodi and Byrne, 2010). Given the relatively conserved structure, and the conserved function of voltage-dependent ion channels, pharmacological tools (e.g.,

channel blockers) that block/actively compete for these channels can be applied to nervous tissue in many model systems to isolate voltage-dependent channels (Rudy, 1988; Lönnendonker et al., 1990; Walsh and Byrne, 1989; Trudeau et al., 1993; Poolos and Johnston, 1999; Dong et al., 2006; Leung, 2012). By using channel blockers, it is possible to characterize the biophysical modifications in B51, 24 h after LTS training.

The main target channels affected by LTS training, in neuron B51, are voltage-dependent Na^+ and K^+ ion channels because of their role in the genesis of action potentials (Na^+ channels are responsible for the depolarization phase and K^+ channels are responsible for the repolarization phase of an action potential). Ca^{2+} channels are not considered a target of LTS training-induced modulation, because previous research has showed that Ca^{2+} channels do not participate in the genesis of B51 plateaus (Plummer and Kirk, 1990). Therefore, the goal of this project was to identify the changes in B51 voltage-dependent channel(s) produced by LTS training, using pharmacological blockers of Na^+ (Tetrodotoxin; TTX), K^+ (Tetraethylammonium; TEA and 4-aminopyridine; 4-AP) and Ca^{2+} (Cobalt; Co^{2+}) channels, respectively (Walsh and Byrne, 1989; Trudeau et al., 1993; Dong et al., 2006). Selective channel blockers have been used previously to isolate the contribution of particular ion conductances underlying neuronal activity in *Aplysia* (e.g., Walsh and Byrne, 1989; Trudeau et al., 1993), as well as in mammals (e.g., Dong et al., 2006; Leung, 2012). The aim of this project was to characterize the biophysical mechanisms by which learning manifests in a decision making neuron in *Aplysia*. These data will help to establish a blueprint for biophysical changes in decision-making neurons, which modulate motor output in the vertebrate nervous system (e.g., Butt and Kiehn, 2003).

Objectives

Objective 1: Investigating the contribution of Na^+ channels to B51 decreased excitability - This objective examined to what extent voltage-dependent Na^+ channels are modulated by a well-established LTS training protocol (Scholz and Byrne, 1987; Cleary et al., 1998; Shields-Johnson et al., 2009) to produce a decrease in excitability in neuron B51.

Objective 2: Investigating the contribution of K^+ channels to B51 decreased excitability - This objective investigated to what extent voltage-dependent K^+ channels are modulated by a well-established LTS training protocol (Scholz and Byrne, 1987; Cleary et al., 1998; Shields-Johnson et al., 2009) to produce a decrease in excitability in neuron B51.

Materials and methods

The experiments were conducted in room 119B in the Center for Science building at Texas A&M University – Corpus Christi. Room 119B contains electrophysiology and behavioral testing equipment. The holding tanks, the tank equipment, and the granted permits involving animal experimental subjects (IACUC) were located in room 119C, also located in the Center for Science building. Texas A & M University and the Institutional Animal Care and Use Committee approved all maintenance, surgical procedures, and experiments.

Animals

Adult *Aplysia californica* (120-190g) were purchased from Marinus Scientific (Garden Grove, California) and South Coast Bio-Marine LLC (San Pedro, Californica)

and were housed in separate aerated containers in filtered artificial seawater (Instant Ocean). The water was maintained at 15°C and animals were kept on a 12 h light/dark cycle in two 529.96 liters aquaria. *Aplysia* were allowed to acclimate to the tanks and recover from shipping for at least three days prior to any manipulation (Wainwright, 2004; Acheampong et al., 2012). All animals were fed strips of dried seaweed (approximately 3 x 19 cm of Emerald Cove® Organic Pacific Nori) three times a week to maintain a healthy weight and a scheduled feeding regime. Animals were food deprived for 48 h before behavioral experiments commenced and remained food deprived throughout the experiment to maintain the same appetitive motivational state (Acheampong et al., 2012).

Preparation of animals for behavioral testing

Parapodectomies – Parapodectomy is a surgical procedure that allows for optimal visualization of the siphon of *Aplysia*, which is critical for measuring withdrawal responses (Scholz and Byrne, 1987; Cleary et al., 1998; Shields-Johnson et al., 2009). Before a parapodectomy procedure was done, animals were buried under ice for 18 min, which anesthetizes the animal temporarily by reducing body temperature (approximately -0.1 °C) (Shields-Johnson et al., 2009). At this point, animals had to be completely relaxed before performing the procedure. Additionally, if an animal inked or secreted opaline under ice it was eliminated from the experiment and returned to the holding tank. After anesthetization, the posterior portions of the parapodia (fleshy appendices that surround and protect the siphon) were clamped at the base with a hemostat to prevent loss of hemolymph during the procedure (Wainwright et al., 2002). Roughly 3 cm of each parapodium were surgically removed, bilaterally, along the base of the body wall (Cleary

et al., 1998). Once the wound resealed, the hemostat was unclamped and the animals were returned to their respective containers in the holding tank to recover for seven days.

Implantation of electrodes - No less than seven days after the parapodectomy, two pairs of Teflon-coated silver wire electrodes (Medwire ®, AG5T) were implanted under the skin in the posterior region of the tail (approximately 1 cm from the tip of the tail and 0.5 cm from the midline; Scholz and Byrne, 1987; Wainwright et al., 2002). Teflon wires were cut (approximately 15 cm) and heat was administered via lighters at each end of the wire (approximately 0.5 cm) until the Teflon melted off. Excess Teflon was scraped off to expose the wire. A multimeter (Fluke 73II) was used to check for resistance and breaks in the wire by taking the leads at one end of the exposed and running the other lead across the wire to the other exposed end (*Fig. 2*).

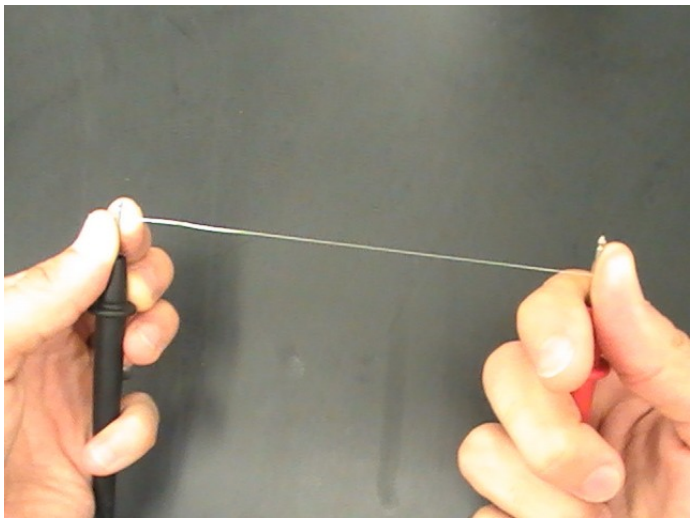

Figure 2. Checking for breaks in Teflon-coated wire using a multimeter (FLUKE 73II).
One exposed end (approximately 0.5 cm) of the wire was then inserted into the tip of a needle ($26G^{3/8}$, Intradermal Bevel) and the remaining length of the wire was coiled 7-8 times around the needle (see *Fig. 3*).

The needle was then attached to a 1 mL syringe by coiling the wire around the tip of the needle, in a spiral fashion, 7-8 times. This was done so that the coils of the implanted electrode could provide an anchor in the connective tissue of the tail.

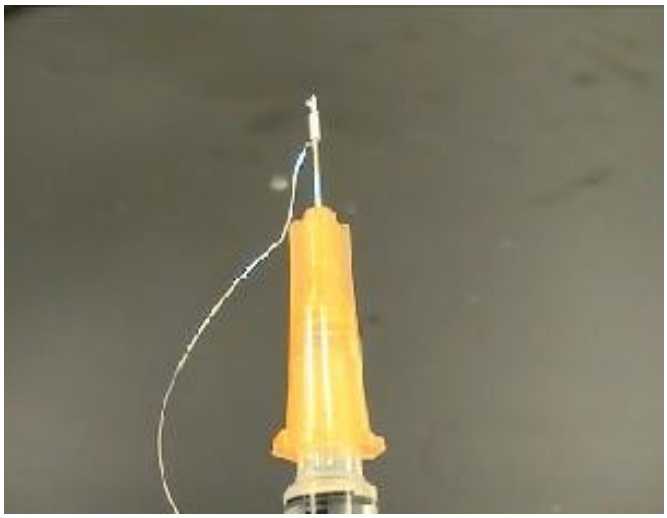

Figure 3. A Teflon-coated silver wire electrode coiled around a needle ready for implantation.

Before implanting the electrodes, animals were made numb with ice in order to anesthetize them for electrode implantation. The site of implantation (left or right side of tail) was randomly selected (coin toss) for each animal and electrodes were spaced roughly 0.5 cm apart. The entire needle was quickly inserted into the tail and then removed (*Fig. 4*). To ensure the wire was properly inserted into the tail, so that a mild electric stimulus could be consistently delivered to evoke tail-siphon withdrawals, the electrodes were lightly pulled (*Fig. 4D*).

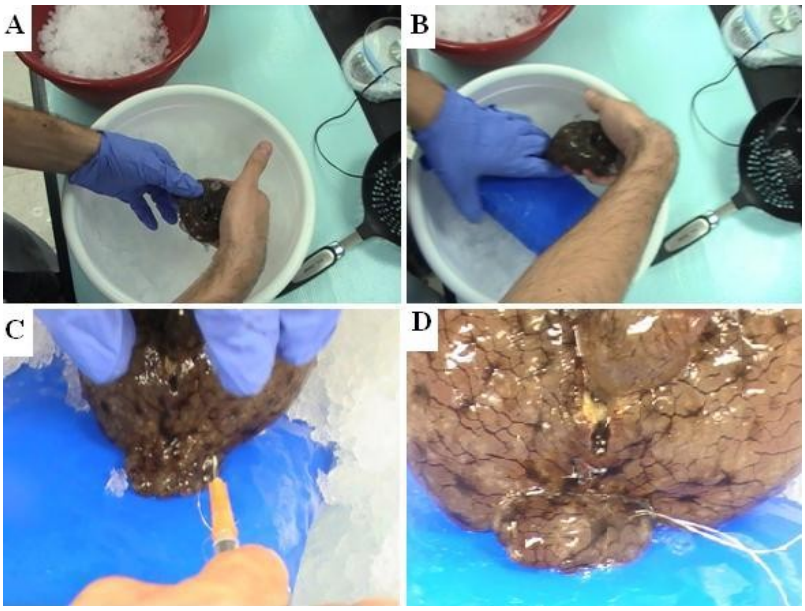

Figure 4. Before being implanted with electrodes, the animal was anesthetized (A), and then the animal's tail was flattened (B) for implantation (C). Afterwards, a slight tug of the electrodes was applied to make sure that they stayed in place (D).

If an electrode came out during this procedure, another electrode was quickly implanted on the designated side roughly 0.5 cm from the other electrode implantation. Implantation occurred 3 days prior to the beginning of behavioral testing (Scholz and Byrne, 1987; Cleary et al., 1998) and the animals were returned to their respective individual containers until pre-testing began (Shields-Johnson et al., 2009).

Behavioral testing

The TSWR and biting behavior were measured before (pre-test) and 24 h after (post-test) training (*Fig. 12*) to establish training-induced changes in these behaviors (for more details see *Measuring TSWR* and *Measuring biting behavior*).

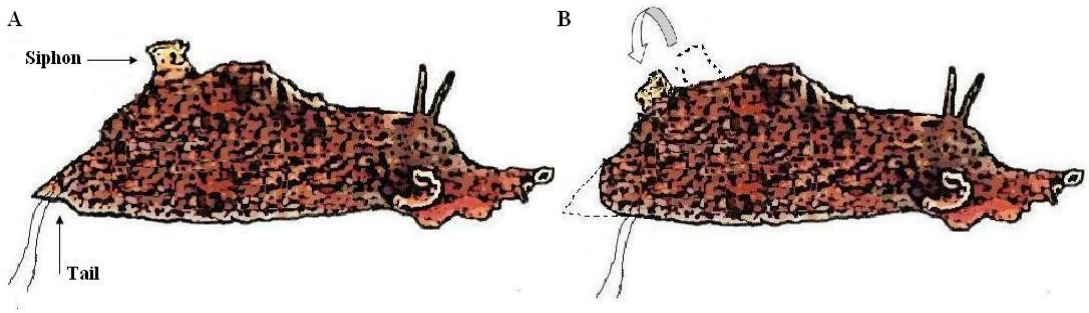

Figure 5. Illustration of the TSWR in *Aplysia*. The siphon is relaxed and visible (A). After a mild stimulus through electrodes, the tail and siphon contract from their original positions (dotted line; B). Modified from Shields-Johnson et al., 2009.

Measuring the TSWR - TSWR was elicited by delivering a mild 20-ms electrical stimulus through the two implanted electrodes (*Fig. 5A,B*; Cleary et al., 1998; Wainwright et al., 2002). The duration of the TSWR served as a measure of the defensive withdrawal reflex strength, and was tested using a well-established protocol (Scholz and Byrne, 1987; Cleary et al., 1998; Acheampong et al., 2012). Animals were first placed in a glass apparatus, which contained 15 ˚C aquarium seawater and was aerated with a stone air pump. The exposed ends of the electrodes implanted in the animal were then carefully inserted between coils of a hookup apparatus that was connected to a stimulator (*Fig. 6*).

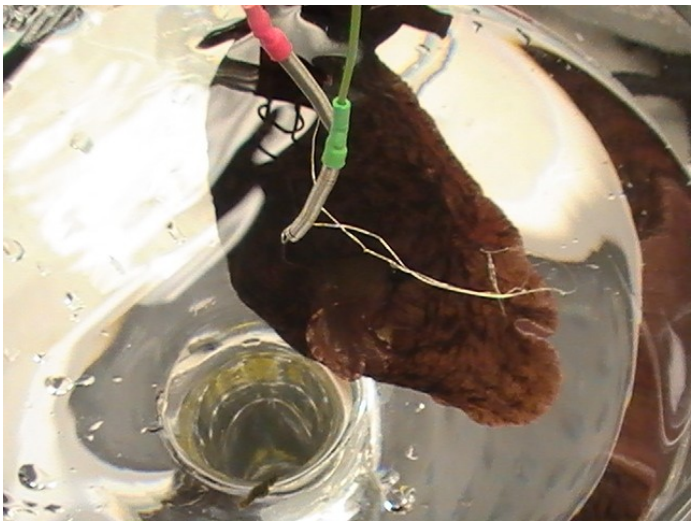

Figure 6. Photograph illustrating the hook-up of implanted electrodes to the testing apparatus.

The length of the wire, with the electrode-attached coil, was then adapted to prevent the coils from pulling out the implanted electrodes if the animal were to move. Prior to obtaining the TSWR duration before training (pre-test), the intensity of the test stimulus, used for the pre- and post-test, was set for each animal as two times the minimal amount of current that elicited a detectable siphon withdrawal (i.e., threshold; Cleary et al., 1998; Wainwright et al., 2002). To obtain the threshold, a mild electric AC stimulus (20-ms duration) was applied. The current intensity was increased by incremental steps of 0.2 mA until a clear contraction of the siphon was elicited. The exact threshold intensity was confirmed using a multimeter (Fluke 73II). Before the pre-test began, animals were acclimated to the glass testing apparatus for another 30 min. During this 30 min, aquarium seawater was carefully changed out using a suction pump, without disturbing the animal, to maintain a temperature of 15ºC.

The TSWR duration was measured prior (pre-test) to the training to establish the baseline TSWR response, and 24 h after the training of the animal (post-test). The pre-test and the post-test TSWR duration procedures were identical and consisted of five test stimuli applied to elicit the TSWR. The duration of the TSWR was measured as the time the siphon began to contract until it began to return to its original position (*Fig. 5*). The stimuli were delivered at 10-min intervals, and the five TSWR responses were averaged. The TSWR duration average was analyzed as a post-test divided by pre-test (ratio) and served as an index to determine the presence of LTS (Cleary et al., 1998; Wainwright et al., 2002). All electric stimuli were delivered by a Variable AC (Variacs) Line Supply (Global Specialties®) and the stimulus duration was controlled by a PulseMaster™ A300 (World Precision Instruments).

Measuring biting behavior – Biting behavior was quantified 30 min after the end of TSWR duration measurements (*Fig. 12;* Acheampong et al., 2012). In order to evoke bites, seaweed extract (SWE) was used as a constant chemical stimulus (Acheampong et al., 2012). SWE was prepared using 300 mL of aquarium seawater placed into a glass beaker with half a sheet of seaweed (approximately 10.5 cm x 19.3 cm) cut into small squares (Shields-Johnson et al., 2009). This procedure guaranteed that all seaweed was dissolved into solution following 30 min of stirring. 30 min after stirring, SWE was separated from solid seaweed using filter paper. 167 mL of the filtered seaweed SWE was then added to 1333 mL of aquarium seawater (8 part aquarium seawater and 1 part filtered seaweed extract). Animals were carefully transferred into the glass testing apparatus containing a concentrated SWE solution that provided tonic chemical stimulation to the rhinophores (chemosensory organs that respond to food stimulus; Kupfermann, 1974) and lips (Brembs et al., 2002; Acheampong et al., 2012). The apparatus was placed on a turntable, allowing for biting behavior of the freely moving animal to be observed from all directions. Once placed in the SWE, biting behavior was measured in a 5-min testing period as the ability of the animal to generate bites, defined as opening of the jaws and a complete cycle of protraction and retraction of the radula (*Fig 7*; Brembs et al., 2002; Lorenzetti et al., 2006).

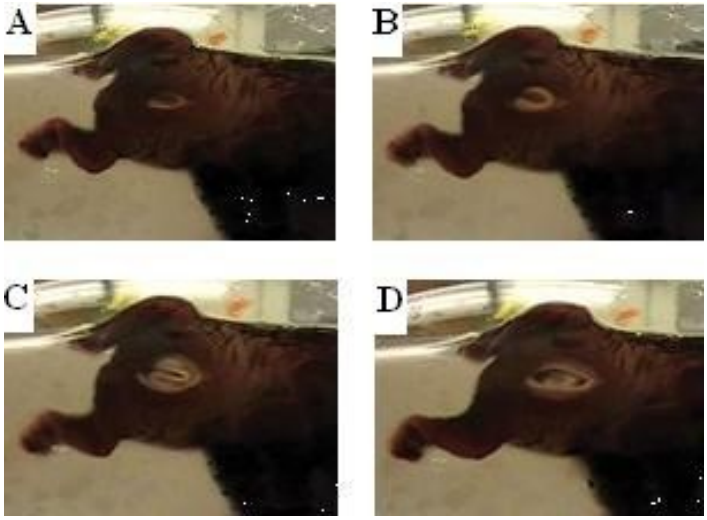

Figure 7. A bite consisting of a complete cycle of protraction (A-B), closure (C) and retraction (D) of the radula.

Biting behavior tests were conducted 30 min after the conclusion of TSWR pre-tests and post-tests (*Fig. 12*). For each animal, the difference in bites (bites in post-test minus bites in pre-test) was calculated and used as an index to indicate changes in biting behavior induced by treatment (LTS trained or untrained controls) (Lechner et al., 2000; Lorenzetti et al., 2006; Acheampong et al., 2012).

Long-term sensitization training

Two groups of animals were used: trained with noxious stimuli and untrained controls (see below). A well-established behavioral protocol was utilized for LTS training (Scholz and Byrne, 1987; Cleary et al., 1998; Wainwright et al., 2002; Acheampong et al., 2012), consisting of four trains of noxious stimuli, each presented at intervals of 30 min (*Fig. 8*).

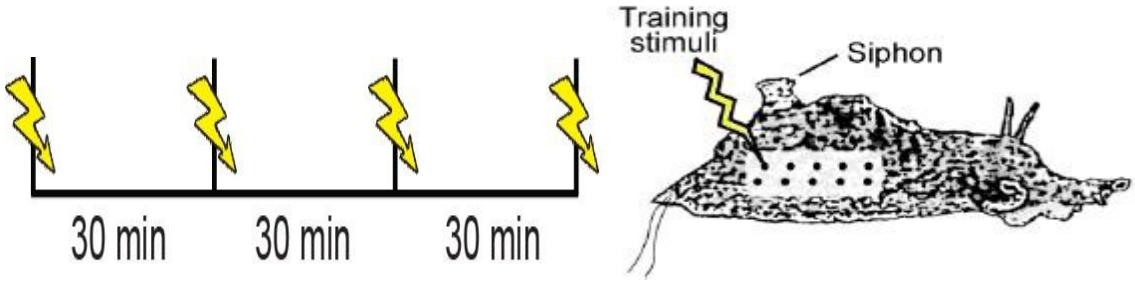

Figure 8. Protocol for LTS training conducted 30 min after feeding pre-test. The yellow "lightning bolts" represents the electric shock administered to the lateral body wall of the animal via a hand-held wand (total of 4 shocks over a 1.5-h time period). *Aplysia* figure modified from Acheampong et al., 2012.

The stimuli used for sensitization training were strong AC electric shocks (60 mA, 500-ms duration; *Fig. 12A*) delivered through a hand-held wand. For each train, 10 stimuli (1 Hz) were administered across the lateral body wall of the side in which the electrodes were implanted. Defensive secretions (i.e., opaline and/or ink) were excreted in response to sensitizing stimuli (Scholz and Byrne, 1987; Cleary et al., 1998; Wainwright et al., 2002). All animals were handled identically, but half did not receive the electric shocks to the body wall (i.e., untrained controls; *Fig. 12B*) (Scholz and Byrne, 1987; Goldsmith and Byrne, 1993; Wainwright et al., 2002). This training protocol was utilized as it produced LTS, which persisted for at least 48 h but less than 72 h (Khabour et al., 2004), and suppressed feeding at 24 h (Acheampong et al., 2012). The animals were selected randomly (coin toss) to be in either the trained or untrained group. The person measuring the behaviors and electrophysiological data was blind to the treatment of the animal.

Criteria for animal use

Several criteria were developed to eliminate animals that did not fully recover from parapodectomy and/or electrode implantation (Acheampong et al., 2012) as well as animals that were already sensitized prior to the treatment. Animals were disqualified if:

1) they generated less than 10 bites in seaweed extract (5 min) before being parapodectomized (Shields-Johnson et al., 2009), 2) they generated less than 5 bites during the feeding pre-test, 3) they exhibited an average TSWR greater than 10 s during the pre-test (see *Measurement of TSWR* for details; Scholz and Byrne, 1987; Goldsmith and Byrne, 1993; Wainwright et al., 2002), 4) they excreted ink and/or opaline before training (Scholz and Byrne, 1987; Wainwright et al., 2002).

Measurement of B51 membrane properties

The protocol that was used to measure B51 membrane properties was derived from Mozzachiodi et al. (2008). Following the post-test for feeding behavior (*Fig.12*), animals were quickly anesthetized by injecting a volume of isotonic $MgCl_2$ through the foot and into the hemocoel equal to 50% of their weight (Mozzachiodi et al., 2008). The isotonic $MgCl_2$ decreased the neuronal activity of the *Aplysia,* which suppressed the animal's pain and muscle contraction. This allowed for the removal of the buccal ganglion from the buccal mass. The buccal ganglion was then pinned onto the Silgard-coated base of a plastic recording chamber, which was filled with a solution of artificial seawater containing high concentrations of divalent cations (Hi-Di ASW). The Hi-Di ASW was utilized to decrease neural activity during the dissection (Byrne et al., 1978; Mozzachiodi et al., 2008) and the composition was (in mM): NaCl 210, KCl 10, $MgCl_2$ 145, $MgSO_4$ 20, $CaCl_2$ 33, and HEPES 10 (pH adjusted to 7.5 with Na0H). The buccal ganglion was then desheathed, allowing access for intracellular recording of neuron B51. One Bn.2,3 nerve was then isolated and was prepared for nerve stimulation, to be used to identify B51, if necessary. B51 responded to the electrical stimulation of Bn.2,3 (4 Hz, 10 V stimulation were applied with 500 ms pulse widths) with an initial inhibitory post-

synaptic potential (IPSP) followed by a plateau potential, making Bn.2,3 an important tool for confirming the identity of B51 (Shields-Johnson et al., 2009). The Hi-Di ASW was then exchanged, using a transfer pipette, for normal artificial seawater (ASW, approximately 20 mL). ASW was utilized as a surrogate for hemolymph, which allowed the experimenter to record the activity of the cells in their natural physiological environment, and was composed of (in mM): NaCl 450, KCl 10, $MgCl_2$ 30, $MgSO_4$ 20, $CaCl_2$ 10, and HEPES 10 (pH adjusted to 7.5 with NaOH). Using a temperature controller during intracellular recordings, the ASW in the dish was maintained at 15°C. B51 was identified based on its anatomical location, size of its soma (*Fig. 9*), and by its ability to produce sustained, regenerative bursts of action potentials (plateau potential; Nargeot et al., 1999a,b; Lorenzetti et al., 2006; Mozzachiodi et al., 2008).

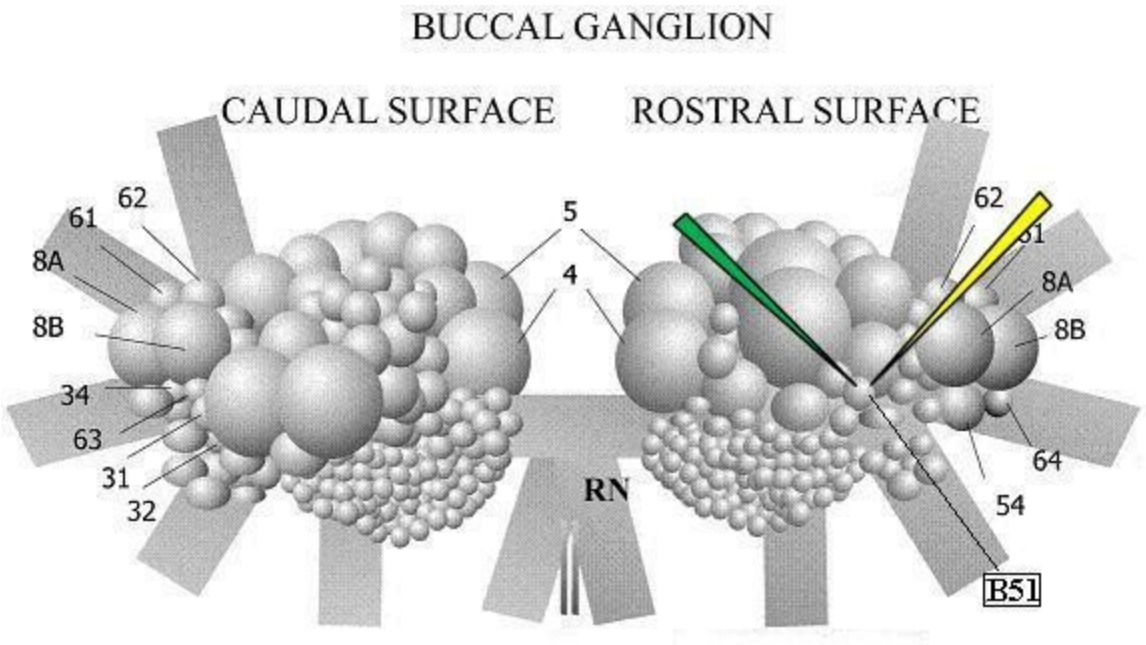

Figure 9. An illustration of the buccal ganglion (only rostral side provides access to B51's soma). The yellow electrode was the stimulating electrode, while the green electrode was the recording electrode.

Standard two-electrode current-clamp was used for intracellular recordings; one electrode injecting current and the second recording activity (Nargeot et al., 1999a,b).

The electrodes were fine-tip glass capillary microelectrodes (World Precision Instruments) and were prepared using a micropipette puller (Sutter Instruments Co.), filled with 2 M cesium methanosulfate. The cesium solution acted as a medium for the injected current and was used so that the voltage responses to injected current were not contaminated by using a K^+-based solutions. Ideally, the two-electrode voltage clamp technique would have been used to analyze the voltage-dependent currents and their modulation by LTS; however the arborization of neuron B51 precludes voltage clamping (Dong et al., 2006). Therefore, two-electrode current-clamp was used under conditions in which Na^+ or K^+ conductances, respectively, dominate B51's voltage response to current injections (i.e. using selective channel blockers; Dong et al., 2006).

Five min after B51 impalement, resting membrane properties (Vm and Rm) and burst threshold, were recorded (Lorenzetti et al., 2006; Mozzachiodi et al., 2008). The Vm was recorded before obtaining the Rm. Both Rm and burst threshold were measured with B51 current clamped at -60 mV. The Rm was measured by injecting 5 nA hyperpolarizing current for 5 s, and was calculated by dividing the change in voltage by the 5 nA of current. (Both the Vm and Rm were expected to be the same between LTS trained and untrained neuron B51 because only excitability (i.e., burst threshold) is affected by LTS training; *Fig. 1A, B, C*). The burst threshold was measured by injecting 5 s of depolarizing current, starting at 5 nA, incrementally increasing by 1 nA until a plateau potential was elicited (Mozzachiodi et al., 2008) or until 30 nA was reached. In order to classify the burst threshold, the criterion was that the activity from the neuron must continue past the 5 s depolarizing pulse (Mozzachiodi et al., 2008).

Preparing channel blockers

Making the TEA, 4-AP and Co^{2+} solutions – TEA and 4-AP were made into a solution, using ultra purified water, at a concentration of 40 mM for 4-AP and 250 mM for TEA (pH = 11.05). Because the solution was very basic, and the ASW that bathes the buccal ganglia must be at 7.5, HCl was added to this solution at a concentration of 25 mM (pH = 9.14). Co^{2+} was made as a separate solution with a concentration of 30 mM (pH = 4.33), in ultra purified water, and had a concentration of 35 mM HEPES to make the solution less acidic (pH = 5.48). The final solution, with 4-AP, TEA and Co^{2+} dissolved in a normal divalent solution, had a final pH of approximately 7.5.

Making the TTX solutions – The $CoCl_2$ was made into a solution of 30 mM. Because both TTX and Co^{2+} are very acidic, the Co^{2+} was dissolved in a 1 M HEPES solution (pH = 7.62) and the final solution had a pH of 5.62. 1mM TTX was made into a solution of 1M HEPES and the pH was brought up to with 1M NaOH. The final solution, with both TTX and Co^{2+} dissolved in a normal divalent solution, had a final pH of approximately 7.6.

Isolation of voltage-dependent Na^+ and K^+ channels

Isolating B51 voltage-dependent Na^+ channels – In order to analyze Na^+ channels in B51 activity, 25 mM TEA (Trudeau et al., 1993; Jacklet et al., 2006) and 4mM 4-AP (Trudeau et al., 1993) were used to block transient and delayed-rectifier voltage-gated K^+ channels, and 15 mM Co^{2+} (Jacklet and Tieman, 2004; Jacklet et al., 2006) was used to block Ca^{2+} and Ca^{2+}-dependent voltage-dependent K^+ channels. After obtaining B51 burst threshold, 300 μL of artificial seawater was taken out of the recording dish. Then 100 μL of the Co^{2+} solution was applied directly onto the desheathed buccal ganglion. Thirty s

later, 200 µL of the TEA and 4-AP solution was added (See *pilot research* in results section for details; A similar procedure was done for *Measuring the effect of LTS training on B51 voltage-dependent K^+ channels*, using the respective channel blockers).

Isolating B51voltage-dependent K^+ channels – In order to analyze the role of K^+ channels in B51 activity, 100µM TTX (Trudeau et al., 1993; Dong et al., 2006), and 15 mM Co^{2+} (Jacklet and Tieman, 2004; Jacklet et al., 2006) was made into a stock solution, used to block voltage-dependent Na^+ and Ca^{2+} ion channels. After obtaining B51 burst threshold, 300 µL of artificial seawater was taken out of the recording dish. Next, 100 µL of Co^{2+} was added to the desheathed ganglion. Thirty s later, 200 µL of TTX was added to the desheathed ganglion, and B51 properties were once again obtained (as described in *Measuring B51 properties after isolating voltage-dependent K^+ channels*).

Internal controls

Internal control for determining the efficacy of TEA, 4-AP and Co^{2+} concentrations – As an internal control, neuron B3 was impaled with a stimulating and recording electrode, and was injected with 5 nA of depolarizing current for 20 ms, incrementally increasing the applied current by 5nA until an action potential was initiated.

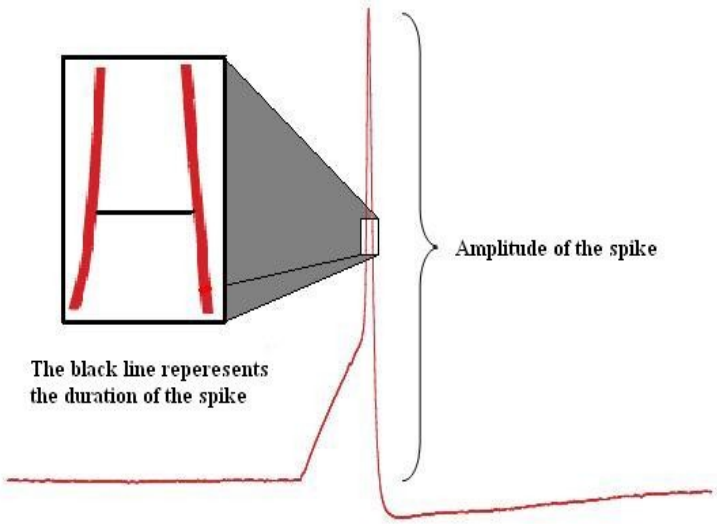

Figure 10. Measuring the spike duration (i.e., spike width) at 50% of the peak amplitude of action potentials in B3.

The spike width (*Fig. 10*) was quantified at 50% of peak amplitude (Antzoulatos and Byrne, 2007). The amplitude of the spike was measured as the peak of the spike to the baseline (*Fig. 10*), and the Vm was current clamped at -55 mV in neuron B3. After obtaining this spike duration in B3, B51 properties were obtained, channel blockers were applied, and then B3 was impaled again and the spike duration was obtained again. This same procedure was done for the second objective (i.e., isolating K^+ channels) of this experiment. The spike durations before and after the channel blocker application were compared to determine if there was an increase in spike duration, which was to be expected, after blocking all K^+ and Ca^{2+} channels (Gingrich and Byrne, 1985; Klein, 1994; Sugita et al., 1997; Phares and Byrne, 2005).

Internal control for determining the efficacy of TTX and Co^{2+} concentrations –

When neuron B3 was impaled after isolating voltage-dependent K^+ ion channels, 20 ms depolarizing pulses were applied up to two times the threshold to elicit a B3 spike before isolating K^+ channels. In some preparations, depolarizing pulses were applied to

B3 until reaching 90 nA, to be certain that spikes were not elicited. It was expected that action potentials could not be elicited; only K^+-dependent depolarizations (Dong et al., 2006).

Measuring B51 properties after isolating Na^+ and K^+ voltage-dependent channels

Measuring B51 properties after isolating voltage-dependent Na^+ channels – For both LTS trained and untrained animals, the Rm, the burst threshold measurements were obtained with neuron B51 clamped at -60 mV. The Vm was measured 5 min after impaling neuron B51.

Measuring B51 properties after isolating voltage-dependent K^+ channels – Five properties were measured after isolating voltage-dependent K^+ channels in LTS trained and untrained B51: Vm, Rm, the amplitude of depolarizing pulses, the input resistance to depolarizing pulses, and the area of depolarizing pulses (intensity and duration of depolarizing pulses are described below). The Rm, amplitude of depolarizing pulses, the input resistance to depolarizing pulses, and the area of depolarizing pulses were obtained with neuron B51 clamped at -60 mV. To measure the amplitude of K^+-dependent depolarizations in B51, 5-second depolarizing pulses of incremental intensities from +5 to +30 nA were used; the range in which plateau potentials were generated (Mozzachiodi et al., 2008; Shields-Johnson et al., 2009). The resistance to the depolarizing pulses (i.e., amplitude divided by the respective depolarizing current, MΩ) was also calculated, giving an indication as to the nature of the voltage-dependent K^+ changes in B51's activity. The area of the depolarizations were then analyzed by calculating the area underlying each depolarizing pulse after adding channel blockers, using ImageTool® (*Fig. 11*).

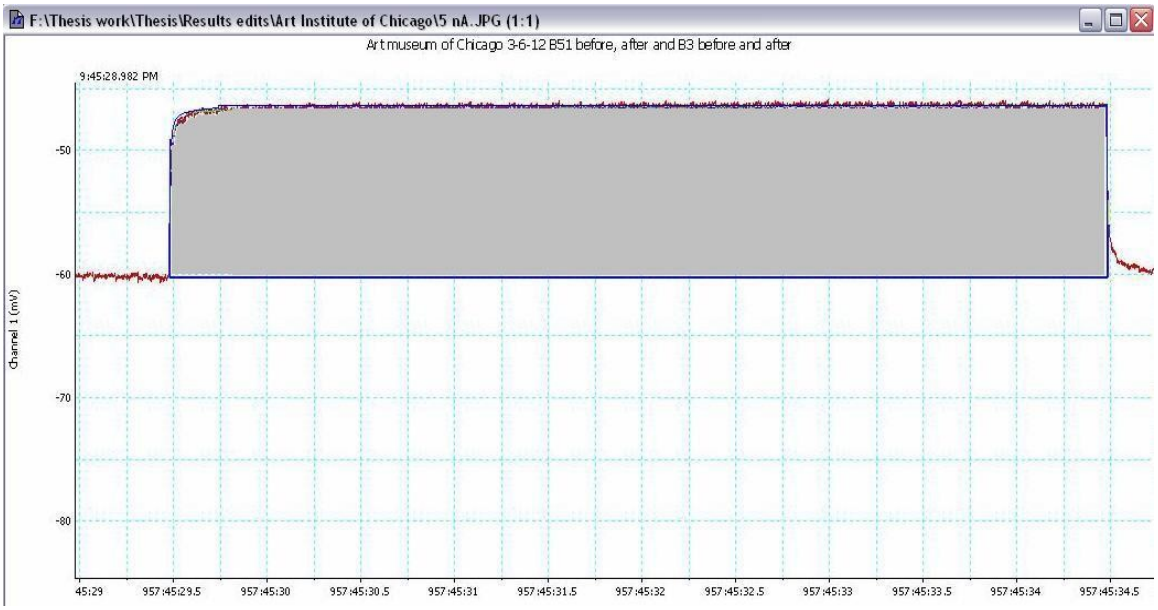

Figure 11. The grey square above represents the area underlying the K^+ -dependent depolarization. All of the points comprising the area were selected (blue outline above) and the ImageTool® software calculated the area.

In order to normalize the areas, and utilize a consistent standard for measuring the areas, the duration of the 5 s depolarizing pulse (*Fig. 11*) was set as 1.0 cm. For each successive depolarization, the 5 s depolarizing pulse duration was re-calibrated as 1.0 cm. After calibrating the standard distance for each trace, a box was drawn to calculate the area underlying each artificially induced depolarization.

Experimental design

The pre-test consisted of measuring baseline TSWR duration and baseline biting behavior. 30 min after recording the baseline biting, the animal was then selected, randomly, to be in the LTS trained (*Fig. 12A*) or untrained grouping (*Fig. 12B*). For trained and untrained groups (*Fig. 12*), the pre-test was the same as the post-test administered 24 h after training. After the biting post-test, the buccal ganglion was prepared for intracellular recording of B51. After obtaining the B51 properties, the whole populations of preparations were split into two groups (*Fig. 12*) to isolate Na^+ and K^+

channels, respectively. The appropriate channel blockers (*Fig. 12*) were applied to the whole ganglia, and B51 properties were obtained once again for *objective 1* and *objective 2*.

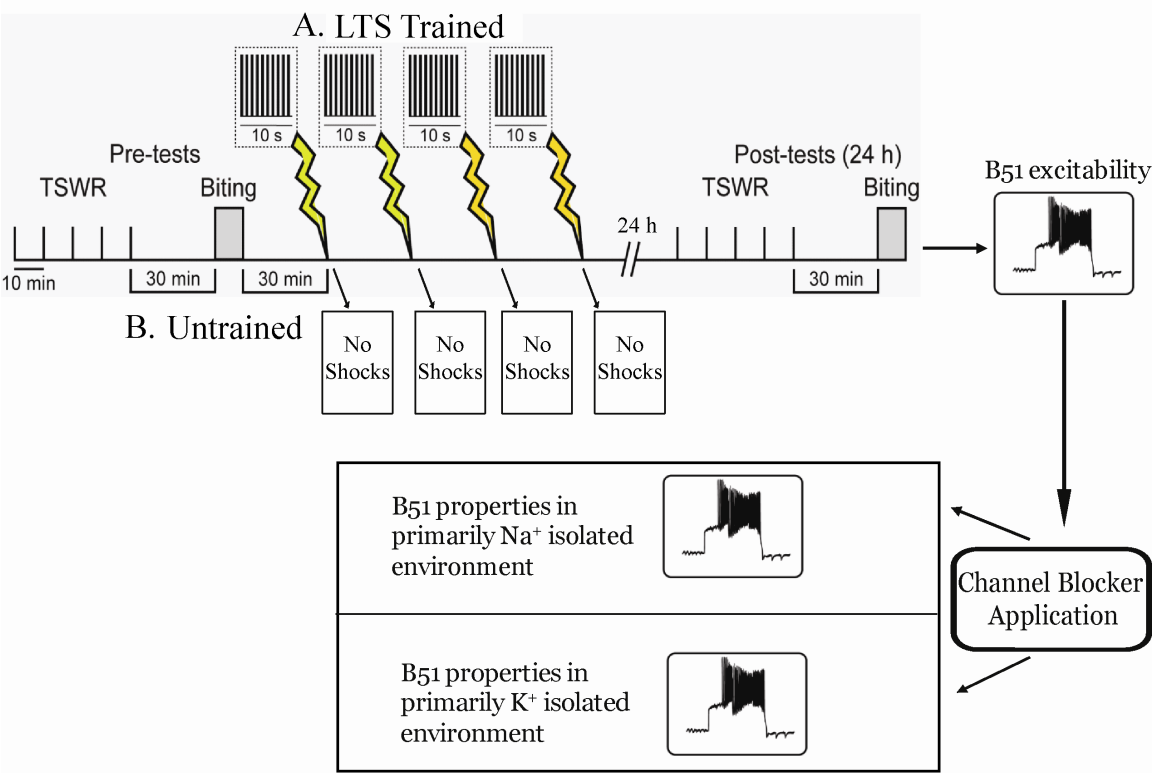

Figure 12. Timeline for the experimental protocol illustrating the behavioral testing, and protocols for LTS trained (A) and untrained (B) *Aplysia*, as well as the measurement of B51 properties. For both objectives, animals from the entire group of experimental animals were assigned to have B51 properties measured in an environment in which Na^+ or K^+ channels, respectively, dominate voltage responses to injected current.

Statistical analysis

All values represent the mean ± standard error mean. All of the behavioral data and B51 electrophysiology measurements, before adding channel blockers, were grouped together for statistical analysis. For the behavioral analyses (i.e., TSWR and biting behavior) and for examination of B51 electrophysiological recordings (i.e., Vm, Rm, and burst threshold), in normal saline, the Mann-Whitney U Test was used to compare sets of data between trained and untrained groups. Once the burst threshold was measured in

normal ASW, preparations were treated with combinations of channel blockers to isolate Na^+ or K^+ channels, respectively. Two separate groups for statistical analysis were made for measuring B51 properties in a primarily Na^+-dependent environment (i.e., Vm, Rm, burst threshold) and K^+-dependent environment (i.e., Vm, Rm, amplitude of depolarizations, area of depolarizations and input resistances to depolarizations), respectively. The Mann-Whitney U Test was used to compare all of the Na^+ and K^+ – dependent properties in B51, between trained and untrained groups. For each training protocol, the amplitude, the areas, and the input resistances of B51 K^+-dependent depolarizations were compared between LTS trained and untrained animals at different current intensities (10 nA, 15 nA, 20 nA, 25 nA). These current intensities were chosen because they are within the range in which both LTS trained and untrained B51 elicited plateau potentials before adding channel blockers. In order to compare B3 spike properties (e.g., spike duration) before and after the addition of channel blockers, in both aims of this experiment, the Mann-Whitney U Test was again used. For all statistical tests, the significant level was set a $p < 0.05$.

Some animals lost one or both wire electrodes prior to one of the post-tests, precluding TSWR measurements for the post-test(s). For these animals, the post-test protocol was followed, but only feeding and B51 properties were measured and included in the statistical analysis. Additionally, more animals were analyzed for the behavioral assessments than for the cellular analysis. This discrepancy was caused primarily by: 1) the inability to find B51 in every preparation, 2) the inability to collect every measurement from each cell. This discrepancy also applies to the B51 properties analyzed after isolating Na^+ and K^+ channels, respectively. Finally, more animals were analyzed for

cellular analysis of B51 than for B51 analysis after isolating Na^+ and K^+ channels, respectively. This discrepancy was caused primarily by: 1) electrodes being pushed out of B51's soma, 2) contractions in the ganglia resulting in the microelectrodes tearing the membrane.

Results

The effects of LTS training on TSWR and biting behavior

A total of 68 animals were used in this study, with 34 control animals and 34 trained animals. For all experimental animals, the animals which received the LTS training displayed a significantly ($p< 0.001$; *Fig. 13A*) enhanced TSWR (1.71±0.13, n=30) compared to the group which did not receive the LTS stimuli (control; 1.19±0.21, n=33). Furthermore, the group which received the LTS stimuli exhibited a significant ($p< 0.001$; *Fig. 13B*) suppression of biting behavior (-7.85±1.13, n=34) compared to the group which did not receive the LTS stimuli (1.14±1.24, n=33).

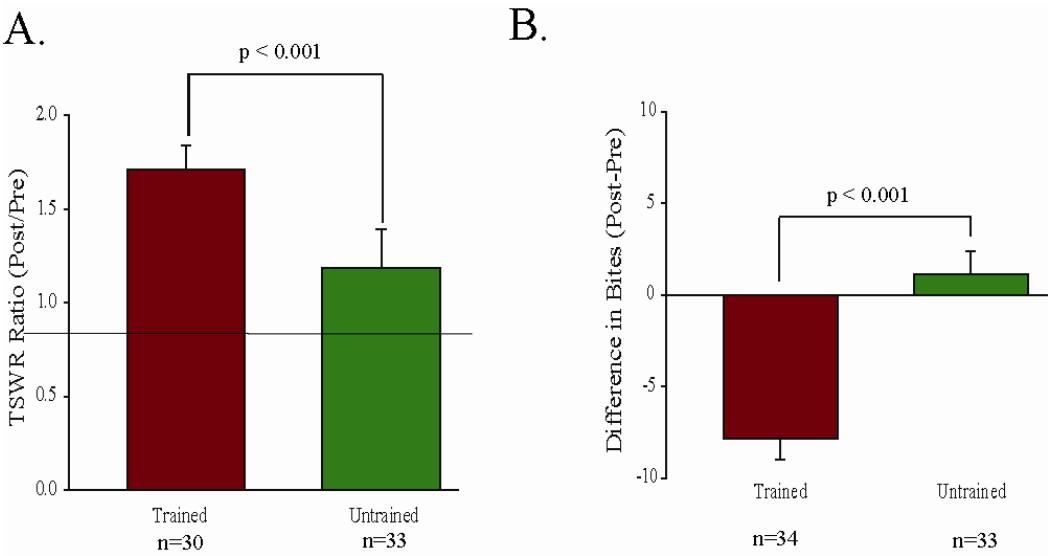

Figure 13. LTS trained animals exhibited an enhanced TSWR (A) and suppressed biting behavior (B) compared to untrained animals.

For one of the feeding tests, the animals' posture (the lips were facing the foot) precluding measuring biting behavior for 3 min and the data was not included in behavioral analysis. This experiment indicates that LTS training induces a suppression of biting behavior that manifests concomitantly with LTS of the TSWR, confirming the findings reported by Acheampong et al. (2012).

Effects of LTS training on B51 membrane properties

Before analyzing the effects of LTS training on pharmacologically-isolated ion channels, it was important to confirm that training caused the previously observed decrease in B51 excitability (Shields-Johnson et al., 2009). LTS training did induce a decrease in B51 excitability, manifested as an increase in burst threshold. The group which received LTS training (20.63±1.5 nA, n=30) displayed a significantly higher (p= 0.005; *Fig. 14B*) burst threshold when compared to the untrained group (14.37±1.35 nA, n=27).

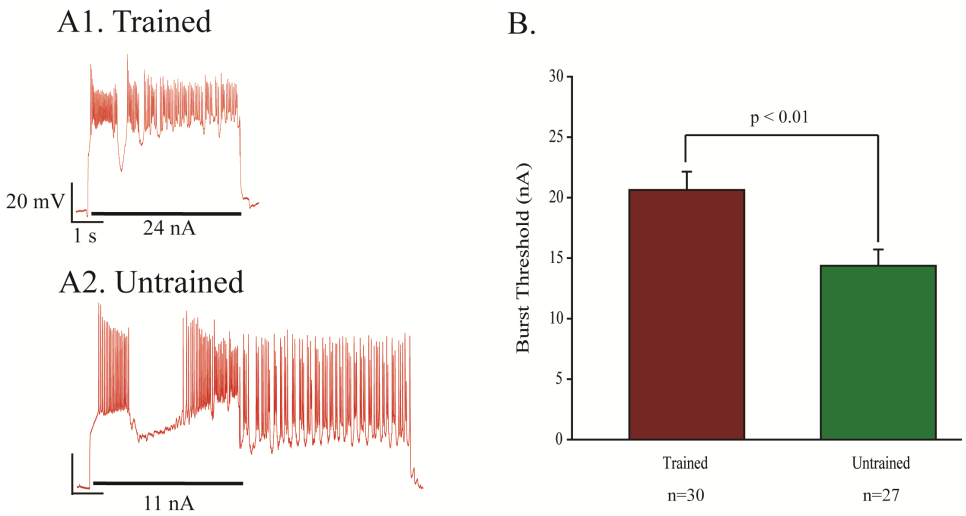

Figure 14. LTS training induced a decrease in excitability manifested as an increase in burst threshold (B). Sample traces illustrate the burst threshold for LTS trained (A1) and untrained (A2) B51.

As previously observed (Shields-Johnson et al., 2009), the Vm before adding

channel blockers in the LTS trained group (-54.87 ±0.93 mV, n=30) was not significantly different (p= 0.917; *Fig. 15A*) from the untrained group (-54.85±0.98 mV, n=27). The Rm was also measured in B51 before adding the channel blockers, and the LTS trained group (2.4±0.07 MΩ, n=29) was not significantly different (p= 0.294; *Fig. 15C*) from the untrained group (2.5±0.1 MΩ, n=27).

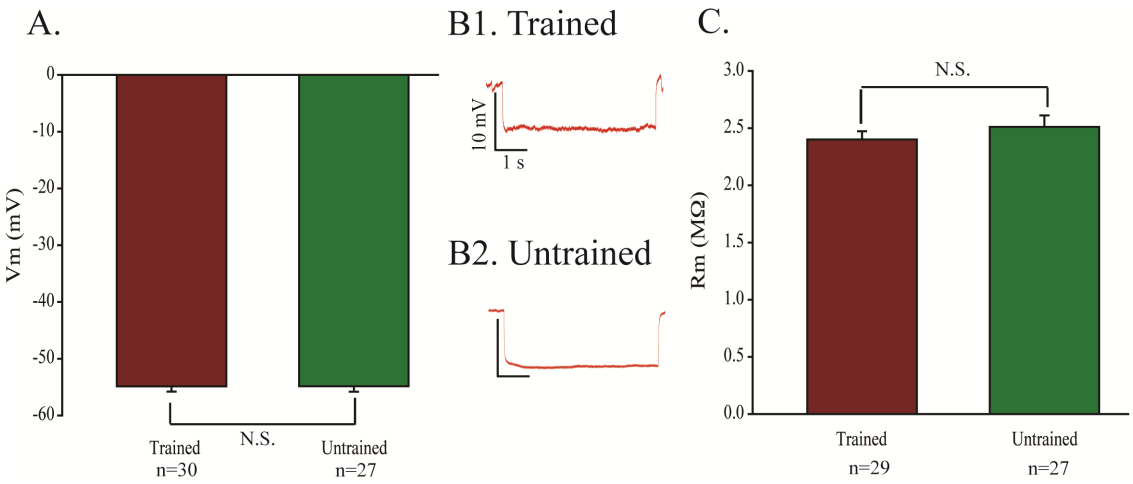

Figure 15. LTS training did not affect Vm (A) or Rm (C) in neuron B51. Traces shown are Rm recordings of LTS trained (B1) and untrained (B2) B51. N.S. denotes not significant.

After ensuring that LTS training suppressed biting behavior and decreased B51 excitability at 24 h, the next step was to ensure the efficacy of the channel blocker combinations for *objective* and *objective 2*. After doing so, B51 membrane properties were once again obtained in an environment in which Na^+ and K^+, respectively, dominated voltage responses to injected current.

Pilot study: Effects of blocking voltage-dependent K^+ and Ca^{2+} channels on elicited spikes in neuron B3

In order to ensure that the concentrations of the channel blockers effectively block K^+ and Ca^2 channels, spikes were elicited in neuron B3 before, and after (*Fig. 16*), adding the channel blockers. The spike duration after adding Co^{2+}, TEA, and 4-AP in neuron B3

(5.442±0.793 ms; n=11) was significantly ($p< 0.001$) greater than the spike duration before adding the channel blockers (3.04±0.618 ms; n=11). The duration of the spike was significantly greater because transient and delayed-rectified K^+ channels, which aide in the return to the RMP by pushing K^+ out of the cell, were blocked with the Co^{2+}, TEA, and 4-AP (Hermann and Gorman, 1981; Walsh and Byrne, 1989; Jacklet and Tieman, 2004), which isolates voltage-dependent Na^+ channels.

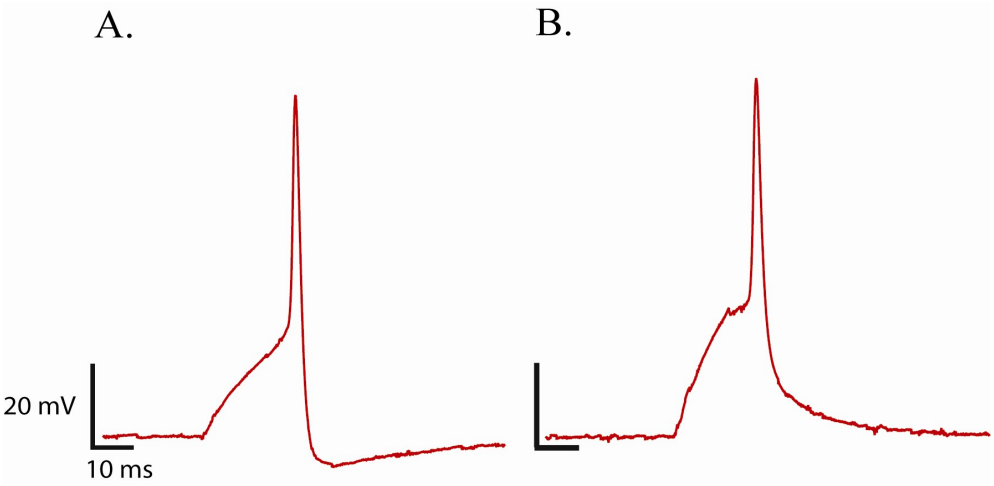

Figure 16. After isolating Na^+ channels, elicited B3 spikes (B) were significantly longer than B3 spike elicited before (A) isolating Na^+ channels.

The contribution of Na^+ channels to B51 properties 24 h after LTS training

In order to determine the contribution of changes in Na^+ channels to B51's decrease in excitability; Rm, Vm, and burst threshold was measured after applying 25 mM TEA, 4mM 4-AP and 15 mM Co^{2+}. There was no significant difference in Vm ($p= 0.26$; *Fig. 17A*) between LTS trained (-49.9±1.76 mV, n=10) and the untrained group (-52.29±1.85 mV, n=7). Also, there was no significant difference in Rm ($p= 0.267$; *Fig. 17C*) between LTS trained (2.65±0.18 MΩ, n=9) and the untrained group (3.60±0.71 MΩ, n=6). These results were anticipated because there was no significant difference before isolating Na^+ channels. For two of the preparations, the cell was lost after recording the

Vm in each group (i.e., LTS trained and untrained). Also, for one of the preparations, one electrode came out of B51 after recording the Rm. When the cell was re-impaled the Rm was a bit higher than the first recording; contributing to the higher Rm error value for un-trained B51.

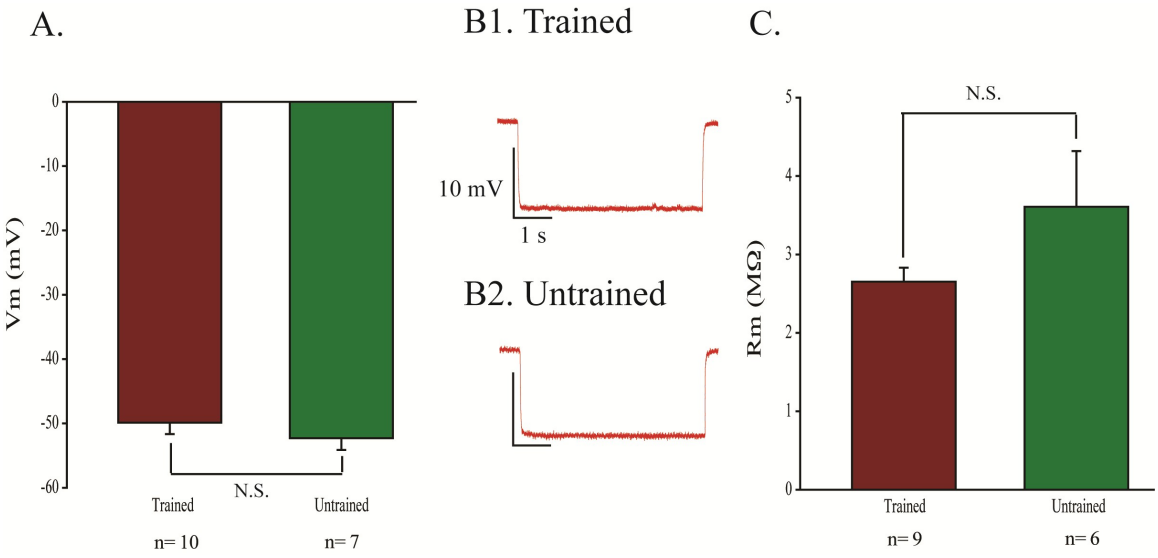

Figure 17. The Na^+ channels of LTS trained B51 were not significantly modulated to change the Vm (A) or Rm (C). Sample traces illustrate Rm measurements of LTS trained (B1) and untrained (B2) B51.

After finding that Na^+-dependent Vm and Rm of LTS trained and untrained B51 were not significantly different, the burst threshold was measured. Interestingly, there was no significant difference in the burst threshold (p= 0.833; *Fig. 18*) between LTS trained (29.5±0.5 nA, n=8) and the untrained group (25.0±5.0 nA, n=5), after isolating Na^+ channels.

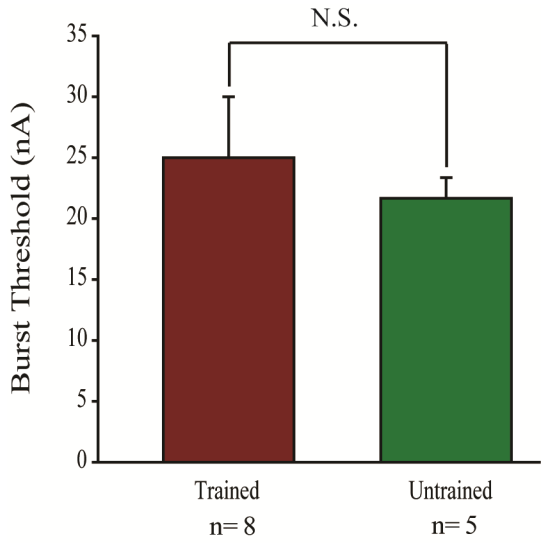

Figure 18. LTS training did not induce a significant change in Na^+-dependent burst threshold.

It must be noted that after isolating Na^+ channels, 90% of the B51 did not elicit plateau potentials. There was, however, some LTS trained (n=1) and untrained (n=1) B51 which did elicit plateaus after the addition of channel blockers. In previous publications, it was suggested that blocking Ca^{2+} channels do not interfere with elicitation of a plateau (Plummer and Kirk, 1990). However, these data suggest that by blocking both Ca^{2+} and K^+ channels, most B51 did not generate a plateau. In light of this finding, a different property was analyzed between LTS trained and untrained B51: the difference in the firing threshold, before and after isolating Na^+ channels (analyzed using the Mann-Whitney U Test). The minimal amount of current necessary to elicit a spike (using the same stimuli for measuring burst threshold) after isolating Na^+ channels was subtracted from the minimal amount of current necessary to elicit a spike before isolating Na^+ channels to obtain the difference in firing threshold. It is important to note that the firing threshold before isolating Na^+ channels could have been considered the burst threshold. The data suggests that the LTS trained B51 (2.30±1.28 nA, n=10) exhibited a higher firing threshold (p=

0.037; *Fig. 19C*) compared to the untrained B51 (-1.29±0.81 nA, n=7).

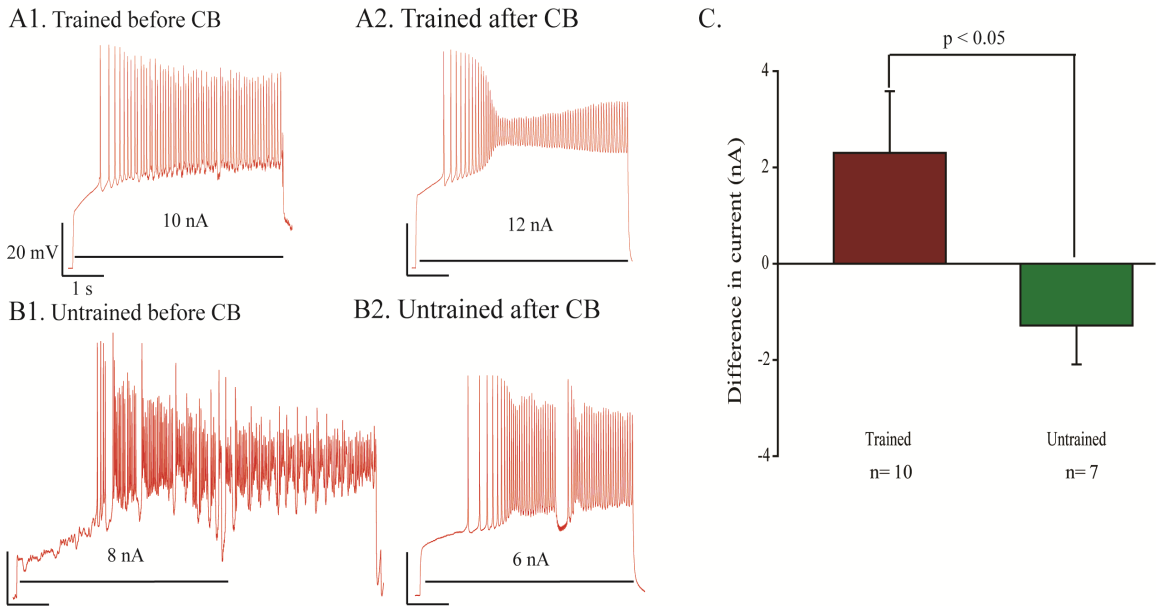

Figure 19. The difference, before and after isolating Na^+ channels, in the firing threshold from LTS trained was significantly higher compared to untrained B51. Sample traces illustrate firing threshold in LTS trained B51 before (A1) and after (A2) isolating Na^+ channels, and also firing threshold in untrained B51 before (B1) and after isolating Na^+ channels. CB denotes channel blocker application.

This finding indicates that when Na^+ channels are isolated in LTS trained B51, more current is necessary to elicit the first action potential. In order to for B51 to produce an all-or-nothing plateau potential, it first must generate the initial action potential, thus initiating the generation of a plateau. This would suggest that the voltage-sensing sub-units of voltage-dependent Na^+ channels are being modulated by LTS training to respond less to depolarizing current injections. The down-regulation of Na^+ channels that causes an increase in the firing threshold may translate into an increase in the burst threshold of LTS trained B51.

Pilot study: The effects of blocking voltage-dependent Na^+ and Ca^{2+} channels on neuron B3 activity

In order to be sure the all voltage-dependent Na^+ and Ca^{2+} channels were blocked with the concentrations used; 20 ms depolarizing pulses were applied before, and after (*Fig. 20*) adding the channel blockers to ensure that action potentials could not be elicited. Action potentials in B3 were consistently elicited between 10-25 nA before adding TTX and Co^{2+}. However, 100% of the depolarizing pulses applied after adding TTX and Co^{2+} (*Fig. 20*) failed to induce an action potential.

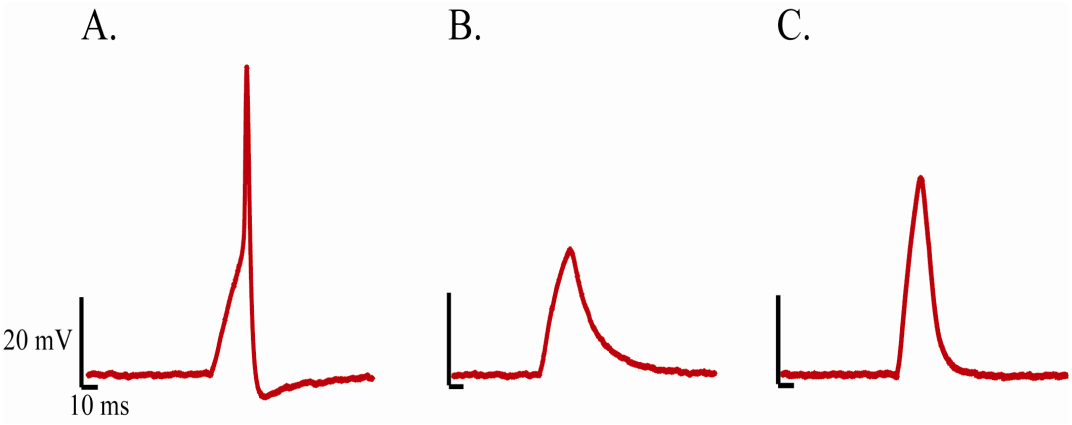

Figure 20. Isolating K^+ channels resulted in the inability to elicit a spike (B) using the same current which elicited a spike (i.e., threshold current) before isolating K^+ channels (A). Doubling the threshold current, after isolating K^+ channels (C), still did not elicit a spike.

The contribution of K^+ channels to B51 properties 24 h after LTS training

After isolating K^+ channels, using well-known channel blockers of voltage-dependent Na^+ and Ca^{2+}, in B51 no action potentials were evoked, and under these conditions depolarizations were measured instead. In order to determine the contribution of changes in K^+ channels to B51's decrease in excitability; Rm, Vm were measured. Additionally, the amplitude, the area, and the input resistances to depolarizing current injections in B51 were measured after applying 100µM TTX and 15 mM Co^{2+}. There was no significant difference ($p= 0.532$; *Fig. 21A*) in Vm between LTS trained -50.1±1.87 mV, n=9) and untrained (-53±1.8 mV, n=10) B51. There also was no significant difference in

Rm (p= 0.316; *Fig. 21C*) between LTS trained (3.88±.35 MΩ, n=7) and untrained B51 (3.6±0.5 MΩ, n=10). Two data points for LTS trained Rm were not obtained; one because the cell was lost, the other because of clogging in the electrode.

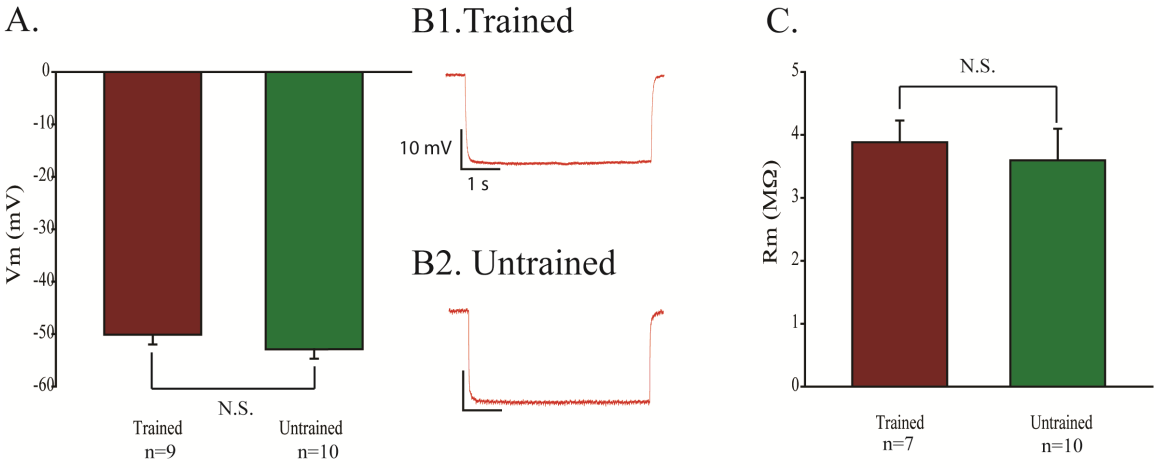

Figure 21. The K^+-dependent B51 Vm (A) and Rm (B) were not significantly affected by LTS training. Sample traces illustrate Rm measurements of LTS trained (C1) and untrained B51 (C2).

These results were anticipated given that Vm and Rm did not differ between LTS trained and untrained B51 before isolating voltage-dependent K^+ channels. After obtaining resting properties, the amplitude of the K^+-dependent depolarizations with increasing current injections (i.e., I-V curve), for LTS trained and untrained B51, were plotted. The I-V curve would better describe the response of voltage-dependent K^+ channels to depolarizing pulses (*Fig. 22*).

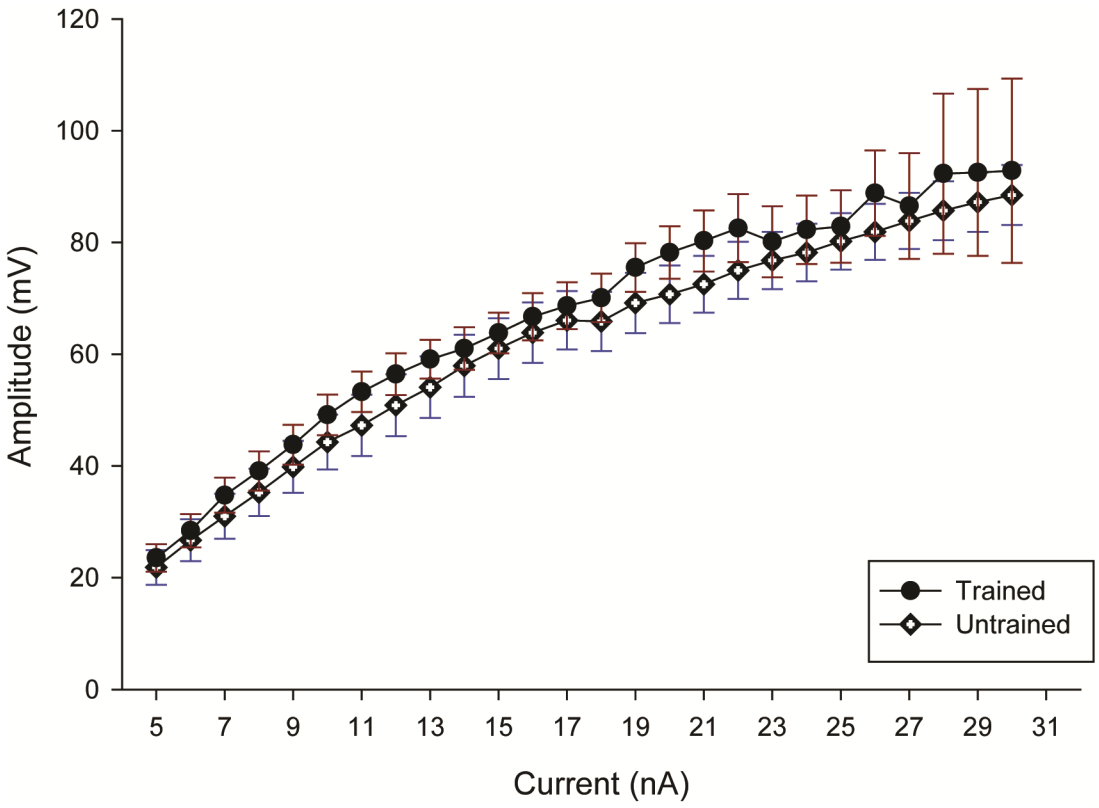

Figure 22. LTS training did not significantly affect the K^+ -dependent amplitude of B51 depolarizations.

Fig. 22 shows the K^+ -dependent voltage responses of LTS trained B51 and untrained B51 to increasing intensities of depolarizing current injections. The data showed that at 10 nA (trained: 49.14±3.64 mV, n=7; untrained: 44.28±4.9 mV, n=9), 15 nA (trained: 63.78±3.63 mV, n=7; untrained: 61±5.43 mV, n=8), 20 nA (trained: 78.2±4.71 mV, n=5; untrained: 71±5.6 mV, n=7), and at 25 nA (trained: 82.84±6.48 mV, n=4; untrained: 80.21±5.1 mV, n=7), K^+ -dependent amplitudes were not significantly different between LTS trained and untrained B51 (for all data points, $p > 0.05$). After obtaining I-V data, the area of K^+-dependent depolarizations from injected current in LTS trained and untrained B51 were measured and compared (*Fig. 23*).

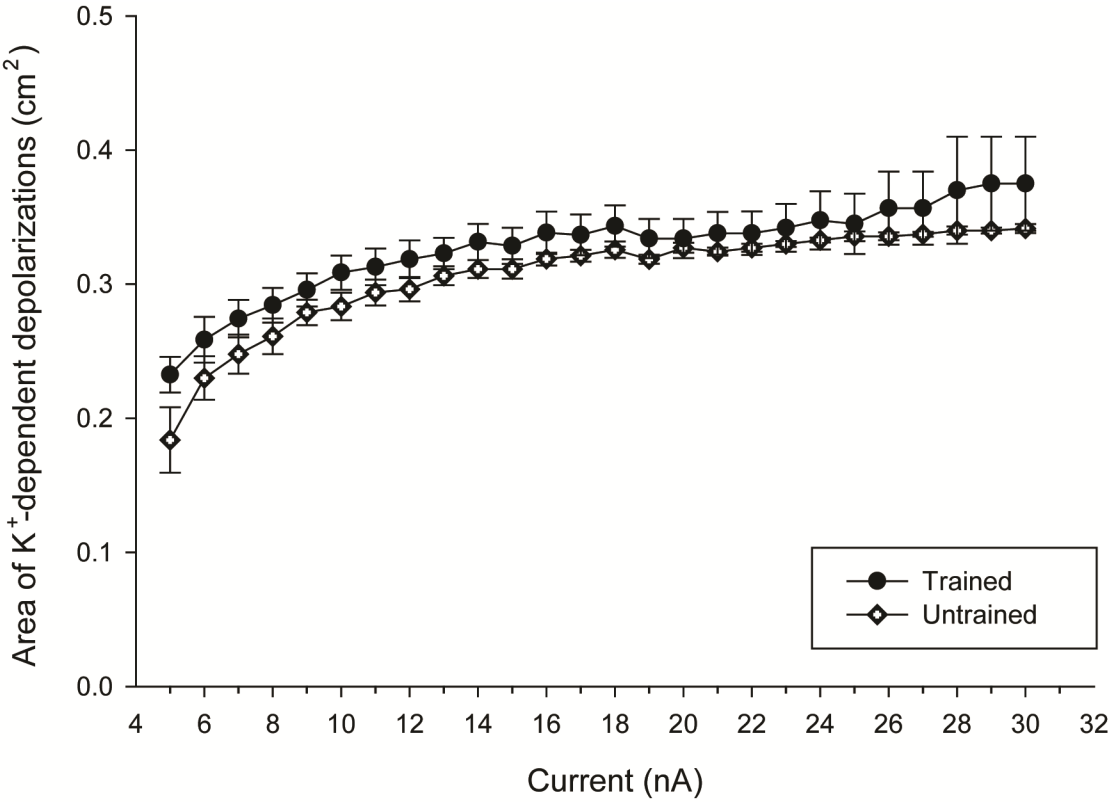

Figure 23. LTS training did not significantly affect the K^+ -dependent area of B51 depolarizations.

Fig. 23 shows the changes in the area of K^+ -dependent depolarization responses, in LTS trained and untrained B51, to incremental intensities of injected depolarizing current. The data shows that at 10 nA (trained: 0.31±0.01 cm^2, n=7; untrained: 0.28±0.01 cm^2, n=9), 15 nA (trained: 0.33±0.013 cm^2, n=7; untrained: 0.31±0.01 cm^2, n=8), 20 nA (trained: 0.33±0.01 cm^2, n=5; untrained: 0.33±0.00 cm^2, n=7), and at 25 nA (trained: 0.35±0.02 cm^2, n=4; untrained: 0.34±0.00 cm^2, n=7), the area of K^+ depolarizations were not significantly different (for all data points, $p > 0.05$) between LTS trained and untrained B51.

Finally, in order to understand the K^+-dependent resistances to depolarizing current injections in LTS trained and untrained B51, an input resistance curve was made.

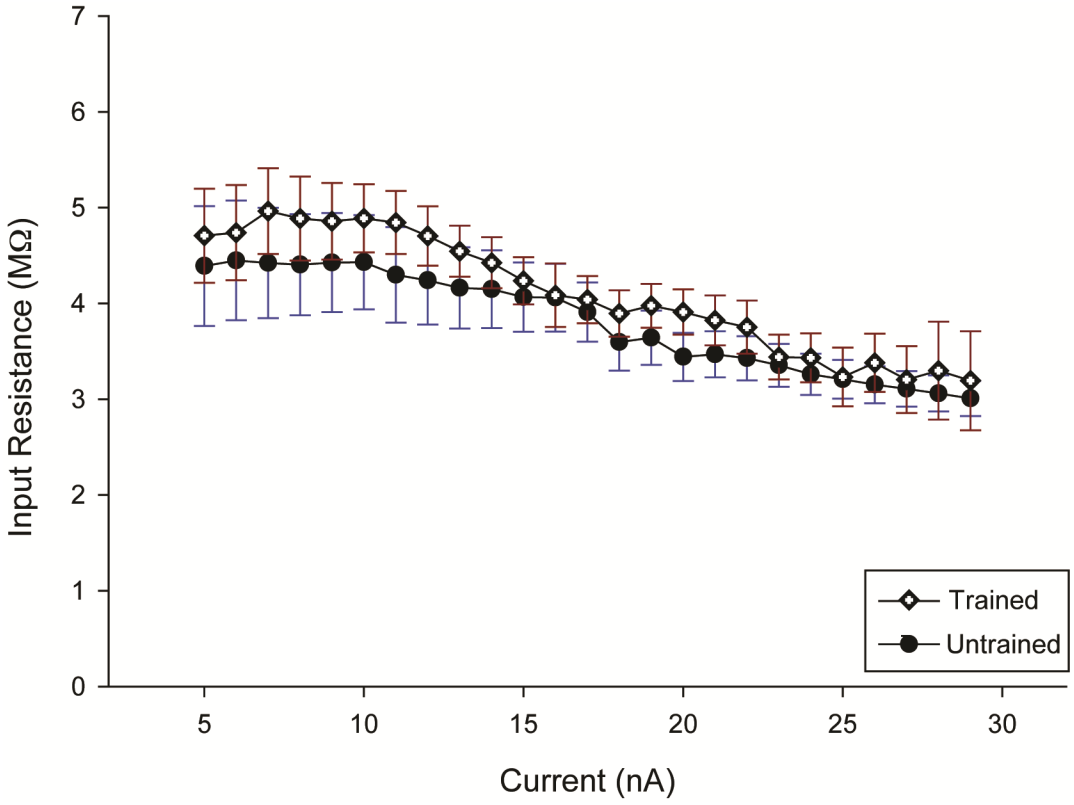

Figure 24. LTS training did not significantly affect the K^+-dependent input resistance to depolarizing current.

Fig. 24 shows the change in the K^+-dependent resistances to increasing depolarizing injected current in LTS trained and untrained B51. The data show that at 10 nA (trained: 4.89±0.36 MΩ, n=7; untrained: 4.4±0.43 MΩ, n=9), 15 nA (trained: 4.24±0.25 MΩ, n=7; untrained: 4.1±0.36 MΩ, n=8), 20 nA (trained: 3.9±0.24 MΩ, n=5; untrained: 3.45±0.25 MΩ, n=7), and at 25 nA (trained: 3.23±0.31 MΩ, n=4; untrained: 3.21±0.2 MΩ, n=7), there was no significant difference (for all data points, $p > 0.05$) in the K^+-dependent input resistances measured in LTS trained and untrained B51.

Overall, the above results indicate that the voltage-dependent decrease in B51 excitability can be caused, at least in part, by an increase in the current needed to activate Na^+ voltage-dependent channels. The finding that voltage-dependent Na^+ channels have a significantly higher firing threshold in LTS trained B51, when compared to untrained

B51, is consistent with the decrease in excitability of LTS trained B51. Using *Aplysia* as a model system, these data have provided an example of how learning can modify a decision-making neuron, at the biophysical level, in a manner which is consistent with learning-induced changes in behavior.

Discussion

The goal of this research was to characterize the means by which a learned fear paradigm (LTS training) modulates an essential behavior (feeding) at the biophysical level, in the decision-making neuron B51. The major findings of this study are as follows: 1) 24 h after LTS training, B51 voltage-dependent Na^+ channels appear to be modulated to be more resistant to depolarizing current and 2) B51 voltage-dependent K^+ channels appear not to be altered by LTS training at the same time point described above.

LTS training induces a suppression of biting behavior concomitant to enhanced TSWR

Much has been learned from studying the effects of LTS training on the TSWR, but the suppression of biting behavior after LTS training has only recently been studied (Hernandez et al., 2011; Acheampong et al., 2012). In general, the results from this experiment show that learned fear is able to modify both defensive and non-defensive behaviors. The enhancement of the TSWR and the suppression of biting behavior have both been observed at 24 h with the LTS training protocol used (Scholz and Byrne, 1987; Cleary et al., 1998; Shields-Johnson et al., 2009) and returned to baseline at 72 h (Hernandez et al., 2011). This study was able to replicate the suppression of biting behavior (Acheampong et al., 2012) seen 24 h after LTS training (*Fig. 13*).

In the neurosciences, many scientists are interested in understanding the effects of fear on the nervous system in both animals and humans (for review, see Rodrigues et al., 2009). Although much headway has been made in understanding how fear manifests in the nervous system (Rodrigues et al., 2004; Adolphs, 2008; LeDoux, 2012), it remains difficult to characterize all of the concomitant cellular learning-induced changes in the nervous system of vertebrate by which behavioral outputs are modified. The nervous system of *Aplysia* allows us to better understand the mechanisms utilized to protect an animal in a state of heightened fear.

LTS training decreases the excitability of B51 at 24 h

Previous research has provided evidence that neuron B51, a key decision-making neuron in the feeding CPG, exhibits a decreased excitability 24 h after LTS training (Shields-Johnson et al., 2009). This decrease in excitability is manifested as an increase in burst threshold (the amount of current necessary to drive B51 into producing its characteristic, all-or-nothing, plateau potential; *Fig. 15*). The results provide compelling evidence that voltage-dependent channels are modulated in B51, given that modifications to resting properties (Vm and Rm) were not observed in LTS trained B51 in previous research (*Fig. 1*) as well as this experiment (*Figs. 14*). It is important to note that past research has shown that operant conditioning learning paradigms for feeding behavior in *Aplysia* induce an increase in the excitability of B51, which is consistent with increased biting behavior observed *in vivo* (Brembs et al., 2002; Lorenzetti et al., 2008). Given these findings, there is compelling evidence that different learning paradigms are able to modulate B51 in a way that is consistent with *in vivo* changes in biting behavior. Finally,

the results from these experiments emphasize the contribution of B51 as a decision-making neuron modulating biting behavior in the feeding CPG.

Modification to firing threshold in voltage-dependent Na^+ channels of B51, 24 h after LTS training

Before the results from this investigation could be critically analyzed, it was necessary to determine the efficacy of the channel blocker concentrations used. The concentrations used in this experiment were chosen to sufficiently isolate K^+ and Na^+ channels, respectively (Hermann and Gorman, 1981; Walsh and Byrne, 1989; Trudeau et al., 1993). The data does show that the blockers concentrations used in *objective 1* of this investigation (*Fig. 16*) effectively blocked K^+ and Ca^{2+} channels. For *objective 2* of this investigation, it was essential to determine the efficacy of the TTX and Co^{2+} solutions, given that previous research has not utilized these two blockers together (e.g., Trudeau et al., 1993; Jacklet and Tieman, 2004; Jacklet et al., 2006). The data shows that 100% of the current pulses applied after isolating K^+ channels did not elicit a spike (*Fig. 20*) in neuron B3. These data offers direct evidence that the concentrations were appropriate to isolate K^+ channels.

The data from *objective 1* provides evidence that LTS training increased the threshold to elicit activity from B51 (*Fig. 19*). The increase in the threshold of B51's Na^+ channels manifested as an increase in the threshold to elicit the first action potential in B51. When Na^+ channels were isolated in untrained B51, the firing threshold appeared lower than before isolating Na^+ channels. This suggests that untrained B51's Na^+ channels respond readily to depolarizing current. However, when Na^+ channels were isolated in LTS trained B51, the firing threshold appeared higher than the firing threshold before

isolating Na^+ channels. These findings indicate that, in the absence of contributions of K^+ or Ca^{2+} channels, LTS training reduces the ability to generate spikes, suggesting long-term modification(s) in the properties (e.g., activation, inactivation) of Na^+ channels expressed in B51. B51 plateau potentials are "all-or-nothing" (Plummer and Kirk, 1990; Brembs et al., 2002; Mozzachiodi et al., 2008), so it is possible that modifications to the firing threshold serve to reduce the production of plateau potentials. A Na^+-dependent reduction in the ability of B51 to fire would postpone the production of an all-or-nothing plateau potential with the consequent decreased likelihood for the elicitation of ingestive BMP, which would ultimately result in the suppression of biting observed *in vivo* following LTS training. Research has shown that in the vertebrate and invertebrate nervous system (e.g., Brons and Woody, 1980; Sanches-Aldres and Alkon, 1991; for review, see Zhang and Linden, 2003; Mozzachiodi and Byrne, 2010), modifications to firing threshold can operate as a physical representation of a memory (i.e., an engram; Mozzachiodi and Byrne; 2010). An engram can be synaptic (i.e., modulation of synaptic strength; Byrne, 1987; Kandel, 2001; Fusi et al., 2005) or can be non-synaptic (i.e., modulation of excitability and firing frequency; Crow and Alkon, 1980; Nargeot et al., 1999a,b), and functions as a memory trace. Even though, in *objective 2*, there was no significant difference in the K^+-dependent B51 properties of LTS trained and untrained B51, were logical given that LTS training-induced decreased excitability of B51.

Based on the data from *objective 2* of this study, when *Aplysia* are taught learned fear using LTS training, voltage-dependent K^+ channels do not seem to be modulated in neuron B51 (*Fig. 22*, *23* and *24*). One logical LTS training-induced modification to voltage-dependent K^+ channels would have been an increase in the outflow of K^+ currents.

However, because the amplitude, input resistance, and the area of K^+-dominated B51 depolarizations were not significantly different in LTS trained B51 compared to untrained B51; the data suggests that K^+ channels are not modulated by LTS training.

Future directions

The next step will be to isolate a factor which triggers B51's decrease in excitability, 24 h after LTS training. One potential candidate for modifying B51's excitability could be serotonin (5-HT). 5-HT is a neurotransmitter which, in *Aplysia*, has been shown to increase in the hemolymph and the nervous system 24 h after LTS training (Levenson et al., 1999). Additionally, artificially increasing the 5-HT in the hemolymph and the nervous system by bath applying *Aplysia* in concentrated 5-HT (Levenson et al., 1999) produces an enhancement of the TSWR similar to that induced by LTS training (Lyons et al., 2006). Given that LTS training induces an enhancement of the TSWR and a suppression of biting behavior at 24 (Acheampong et al., 2012; Hernandez et al., 2011), and 5-HT mediates the enhancement of the TSWR (Levenson et al., 1999; Lyons et al., 2006); it was hypothesized that 5-HT would contribute to the suppression of biting behavior. However, 5-HT bath application, which produced LTS, did not significantly suppress biting behavior, nor did it alter B51 properties (Hernandez et al., 2011). These previous findings suggest that 5-HT does not mediate the LTS training-induced suppression of feeding and that multiple biochemical pathways are concomitantly activated in response to sensitizing stimuli to modulate distinct neural pathways in *Aplysia*.

Another biochemical pathway that might mediate the effects of LTS on biting behavior in *Aplysia* is the nitric oxide (NO) pathway (for review, see Susswein and Chiel,

2012). NO is a biological regulator that has been suggested to initiate biochemical cascades, which in turn induce plastic changes in neurons (Katzoff et al., 2006). These neuronal changes have then been suggested to lead to downstream changes in behavior. Research shows that when an *Aplysia* was injected with a NO donor into the hemocoel, the animal did not attempt to bite in response to food stimulation 24 h after treatment (Katzoff et al., 2006). Furthermore, previous research has revealed that NO plays a significant role in the induction of another form of LTS (i.e., LTS of nocioceptive sensory systems; Lewin and Walters, 1999). Additionally, NO is responsible for initiating a cyclic GMP (cGMP) -dependent protein kinase G (PKG) pathway that underlies LTS of nocioception (Lewin and Walters, 1999). Therefore, LTS training may trigger an increase in NO levels in the hemolymph and nervous system, which could ultimately exert a suppressive effect on biting. In order to determine the role of NO in the suppression of biting behavior observed after LTS training, a putative NO synthesis inhibitor (i.e., L- N^G-nitro-L-arginine, L-nitro; Lewin and Walters, 1999) can be injected into *Aplysia* before LTS training. If NO plays a pivotal role in LTS training-induced suppression of biting behavior, L-nitro would prevent the suppression of biting behavior and preclude the decrease in B51 excitability, seen 24 h after LTS training. Another future direction could be to apply a putative NO donor (i.e., *S*-nitroso-*N*-acetyl-penicillamine, SNAP; Katzoff et al., 2006; Miller et al., 2011b) to neuron B51 to try and mimic LTS training-induced decrease in excitability. If NO does play a role in modulating B51 excitability, SNAP would induce a decrease in excitability of neuron B51.

If NO does induce a decrease in excitability of B51, the following changes to voltage-dependent Na^+ channels could be responsible: 1) NO acts through cGMP to

activate PKG (Lewin and Walters, 1999) to phosphorylate and modify the voltage-sensing domain of Na^+ channels (i.e., S4 domain of alpha subunit; for review, see Bezanilla, 2008) to be more resistant to depolarization of the membrane, 2) NO initiates a cAMP-PKG-dependent pathway resulting in a PKG catalytic subunit decreasing transcription (Lewin and Walters, 1999) of genes coding for production of voltage-dependent Na^+ channels. The latter hypothesis would result in fewer voltage-dependent Na^+ channels which are able to respond to the depolarization of the membrane. Both of the proposed changes to voltage-dependent Na^+ channels could contribute to the increase in the firing threshold of Na^+ channels in LTS trained B51.

Conclusions

Neurons that exhibit decision-making properties have been identified in several model organisms, including humans (Schall, 2003; Rangel et al., 2008). However, the cellular mechanisms underlying decision making in all organisms remains elusive due to the extremely complex circuits of vertebrates. In order to bypass the constraints of vertebrate neurophysiology, the science community has consistently looked towards the well-characterized circuits of invertebrates; more specifically those of *Aplysia californica*. This research provides insights into the biophysical substrates of learning-induced changes in a decision-making neuron in *Aplysia*.

Following the identification of LTS training-induced changes in B51 Na^+ and K^+ currents, future experiments will focus on characterizing the biochemical pathway(s) (e.g., second-messenger cascades) responsible for such changes. Research has

consistently shown that the underlying physiological and biochemical mechanisms utilized in the nervous system are homologous through evolution (e.g., Nemoto et al., 1986; Kandel, 2001), making the characterization of engram formation in the invertebrate nervous system important for understanding the complexity of engrams in the vertebrates, including humans.

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