ROLE OF THE CYCLIC GUANOSINE MONOPHOSPHATE PATHWAY ON THE BEHAVIORAL AND NEURONAL PLASTICITY INDUCED BY AVERSIVE STIMULI IN THE SEA HARE, *APLYSIA CALIFORNICA*

A Thesis

by

RUMA CHATTERJI

BS, Clarion University, 2014

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

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Committee Member

August 2017

ABSTRACT

The ability to make decisions when in a state of trepidation is a universal and crucial component of organism survival. Studying behavior and the underlying cellular mechanisms in parallel is needed, but rarely achieved due to the complexity of the nervous system and the difficulty to link behaviors to cellular substrates. Therefore, the goal of this project was to examine both behavioral and neuronal plasticity using the marine mollusk *Aplysia*, an organism with quantifiable behaviors controlled by well-characterized neural circuitry. In *Aplysia*, exposure to aversive stimuli causes a learned suppression of a non-defensive behavior (i.e., feeding) and a learned enhancement of a defensive response (a form of learning called sensitization) of the tail-siphon withdrawal reflex (TSWR). Correspondingly, at the cellular level, exposure to the aversive stimuli causes a decrease in excitability of B51, a decision-making neuron controlling feeding, and an increase in excitability of tail sensory neurons (TSNs) responsible for the TSWR. The Cyclic Guanosine Monophosphate (cGMP) pathway dependent on protein kinase G (PKG) is involved in learning-dependent behavioral and neuronal plasticity associated with non-defensive and defensive responses in *Aplysia*. The aims of this thesis were: 1) to investigate the role of PKG in feeding suppression and sensitization *in vivo* and 2) to examine the role of PKG in neural correlates underlying feeding suppression via B51 excitability and sensitization via TSN excitability *in vitro*. Selective inhibitor KT5823 was used to block PKG activity. Findings from *in vivo* experiments indicate that KT5823 did not prevent either feeding suppression or sensitization induced by aversive stimuli. Concurrently, *in vitro* results determined that KT5823 did not prevent the learning-induced decreased excitability of B51 and also did not prevent the learning-induced increase in TSN excitability. These results suggest that PKG may not contribute to the

behavioral or neuronal plasticity induced by aversive stimuli in *Aplysia*. Future directions include investigation of other potential downstream targets such as cyclic-nucleotide gated ion channels and phosphodiesterases.

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The culmination of this thesis project is only just the beginning for my future endeavors, and I could not have dreamed of a better platform to have launched my graduate career from. To my mentors, friends and family that have all been a part of this adventure, thank you- I truly appreciate each and every one of you.

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INTRODUCTION

The ability to learn helps organisms to survive and adapt to a constantly changing environment. Moreover, the capability to store and retain information over time is fundamental to an organism's existence (Peeke and Petrinovich, 1984; Christoffersen 1997; Carew et al., 2000; Kandel et al., 2014). Neuroscientists explore the brain to determine the cellular mechanisms involved in behaviors. Extensive work has demonstrated that memory traces can be characterized at the cellular level. For example, researchers demonstrated sound production and vertical head movement in conjunction with different cellular activity in the brain of quail-chick chimeras (Okano et al., 1997) (Figure 1). They investigated how acoustic isolation, resulting from environmental influences, may result in neuronal changes.

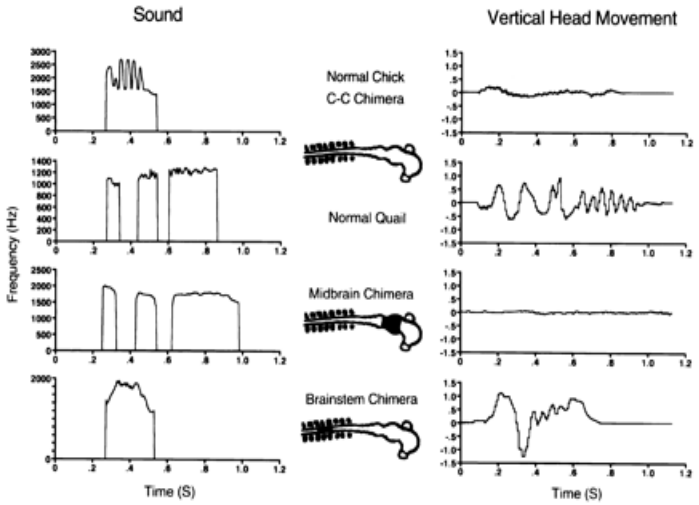


Figure 1 Sound production and head movement with accompanied schematic of brain transplant

This example showcases the behavioral and its associated neuronal activities that are required for survival. Additionally, behaviors are categorized into two groups: defensive and non-defensive. When exposed to aversive stimuli, organisms increase their defensive responses and decrease non-defensive responses (e.g., Timbergen, 1951; Gillette et al., 2000; Jing and Gillette, 2000, Acheampong et al., 2012). For example,

young macaques coo to get their mother's attention when separated from them but stop cooing when threatened to minimize potential predator risk (Kalin et al., 1991). Invertebrates such as *Aplysia californica* also demonstrate these behavioral changes when exposed to an aversive stimulus. Since *Aplysia* have a well-characterized neural circuitry, it is possible to examine the mechanisms responsible for behavioral changes at the cellular level. The scope of this study aimed to understand the behavioral changes that occur in association with aversive stimuli and to investigate the molecule(s) that is responsible for modulating those changes to develop a comprehensive understanding of learning and memory.

The Model Organism: Aplysia californica

To study learning and memory, the model organism used for this project was the marine mollusk, *Aplysia californica* (Figure 2).

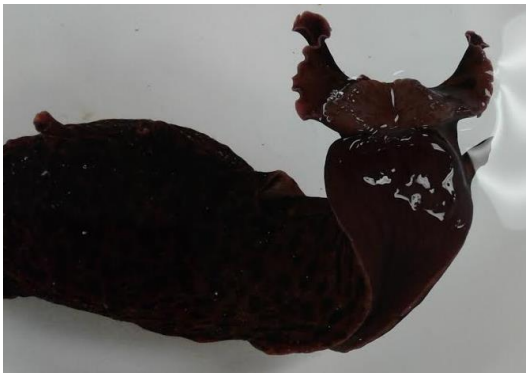


Figure 2 *Aplysia californica*

This species has important ecological implications as it is a primary foraging herbivore and is a food source for predators such as the spiny lobster, *Panulirus interruptus* (Owen, 1980). Furthermore, *Aplysia* behaviors are easily quantifiable, such as their feeding and defensive withdrawal reflexes. *Aplysia* have been extensively used in the field of learning and memory (e.g. Kandel, 2001) as the large neurons (Figure 3) in its ganglionic nervous system enables us to mechanistically link behaviors to the underlying neural circuits (Byrne et al., 2009).

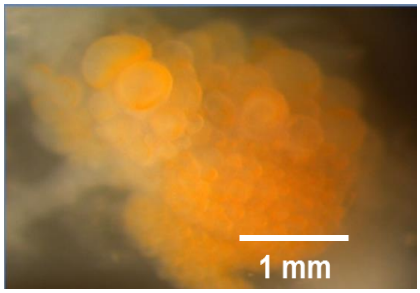


Figure 3 Ganglion of *Aplysia californica*

Behavioral and Neuronal Plasticity: A Parallel Perspective

In *Aplysia*, both behavioral and neuronal plasticity can be explored. Plasticity is when behavioral modifications are achieved through changes in neuronal function manifesting in two main forms: synaptic and intrinsic plasticity. Synaptic plasticity is a form of plasticity where the strength of the synaptic connections varies depending on the exposure of the synapse to different levels of activity (Kandel et al., 2001). While this type of plasticity is highly useful to study, there have been further investigations that learning has influences on intrinsic neuronal excitability (Antonov et al., 2001; Zhang and Linden, 2003 Mozzachiodi et al., 2010; Mozzachiodi and Byrne, 2017). In general, ionic channels can experience several changes that lead to modifications in neuronal membranal properties such as burst threshold, input resistance and resting membrane potential (Zhang and Linden, 2003; Mozzachiodi and Byrne, 2010). Intrinsic neuronal plasticity is important to explore as changes resulting from exposure to various experiences could directly engrain in the memory trace itself (Mozzachiodi and Byrne, 2010). Conclusively, studying the behavioral changes can be achieved through *in vivo* experiments by directly observing the organism while intrinsic neuronal plasticity responsible for these behavioral changes can be studied via *in vitro* experiments. A well-rounded understanding of how an organism retains information in the form of memories requires studying both behavioral changes and accompanying neuronal changes.

Examining both forms of plasticity in the form of learning and memory can be difficult but is possible in organisms such as *Aplysia*.

Learning in Aplysia

Aplysia exhibit both associative and non-associative learning. Associative learning can be further subdivided into classical conditioning and operant conditioning. Classical conditioning involves a relationship between a predictive stimulus and a subsequent event (e.g. Mozzachiodi and Byrne, 2010). Operant conditioning occurs when a reward or punishment is applied to a behavior and subsequently increases or decreases the behavior intensity (Skinner, 1938).

Aplysia also demonstrates non-associative learning, such as habituation (where the response to a repeated stimulus diminishes) and sensitization (where the response amplifies from repeated exposure to a usually noxious stimulus) (Hawkins et al., 1998). These learning processes can manifest into both short-term and long-term memory (e.g. Rankin and Carew, 1986).

Exposure to an aversive stimulus causes changes in both defensive and non-defensive behaviors in *Aplysia.* When the animal is exposed to an aversive stimulus, it increases the intensity of the defensive responses, i.e., displays sensitization (Acheampong et al., 2012). In addition, exposure to aversive stimuli causes a concurrent decrease in non-defensive behaviors such as feeding, as the need to implement such a behavior during times of stress is not needed (Acheampong et al., 2012).

This learning depicted by *Aplysia* that is assessed at the behavioral level as a response to sensitizing stimuli can also be assessed in the isolated *Aplysia* ganglia (Zhang et al., 1994). Together, analysis of both behavioral and cellular adaptations allows a

parallel insight into the learning that occurs when *Aplysia* is exposed to a sensitizing stimulus. Described below are the behaviors that were studied for this thesis along with the neural circuitry modulating these behaviors.

Non-defensive Behavior: Feeding

Feeding in *Aplysia* is a well-characterized behavior involving a chemotactic response to seaweed, its preferred food source (Kupfermann, 1974). Once oriented to the food source, the stereotyped motor typic response involves the protraction, opening and closing, and finally the retraction of the radula (see Figure 4). Analogous to the TSWR and sensitization, feeding in *Aplysia* also portrays learning that is dependent on duration of stimulus exposure (Acheampong et al., 2012). Exposure to aversive stimuli results in feeding suppression (Acheampong et al., 2012; Shields-Johnson et al., 2013).

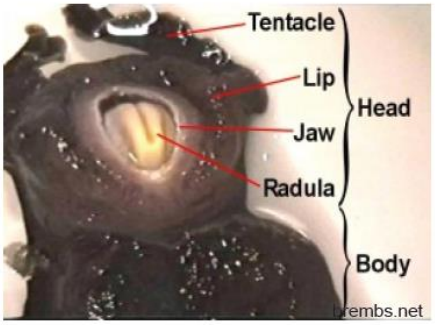


Figure 4 *Aplysia californica* head (brembs.net)

Feeding behavior is controlled by a central pattern generator located in the buccal ganglion (Kupferman, 1974; Mozzachiodi et al., 2013) (See Figure 7). Within the buccal ganglion is a motor neuron B51 which constitutes to the buccal motor patterns (BMP) (Plummer &Kirk, 1990). We can artificially induce activity in neuron B51 by subjecting it to either depolarizing or hyperpolarizing currents (Nargeot et al., 1999; Nargeot and Simmers 2011; Mozzachiodi et al., 2013). When B51 is depolarized, it contributes to ingestive buccal motor patterns whereas when it is hyperpolarized it instigates egestive

buccal patterns (Nargeot et al., 1999; Shields-Johnson et al., 2013; Weisz et al., 2017). This information suggests that the neuron B51 is not only sufficient but necessary for the elicitation of a bite *in vitro* (Nargeot et al., 1999). When the neuron is exposed to sensitization training there is a decrease in B51 excitability associated with decreased number of BMPs. This is similar to the behavioral counterpart where the decrease in excitability is analogous to the animal taking fewer bites (Shields-Johnson et al., 2013).

Defensive behavior: Tail-Siphon Withdrawal Reflex (TSWR)

Aplysia exhibit many defensive responses when subjected to an aversive situation such as the tail-siphon withdrawal reflex (TSWR) (e.g., Goldsmith and Byrne, 1993). This behavior constitutes a reflex that involves the contraction and the subsequent relaxation of the tail and siphon when the animal is exposed to an aversive stimulus (Hawkins et al., 2006).

Sensitization or repeated exposure to an aversive stimulus, such as an electrical shock mimicking a predator attack, enhances the TSWR response (Scholz and Byrne, 1987; Cleary et al., 1998). The duration of this reflex can then be quantified and compared as a measure of sensitization. The memory that is sustained can differ depending on period of stimulus exposure, ranging from short-term time periods to long-term time periods (Cleary et al., 1998; Byrne and Hawkins, 2015).

Within the well-characterized neural circuitry of *Aplysia* is the left and right pleural-pedal ganglia containing tail sensory neurons (TSNs) responsible for the TSWR (Walters et al., 1983) (Figure 5 and 7).

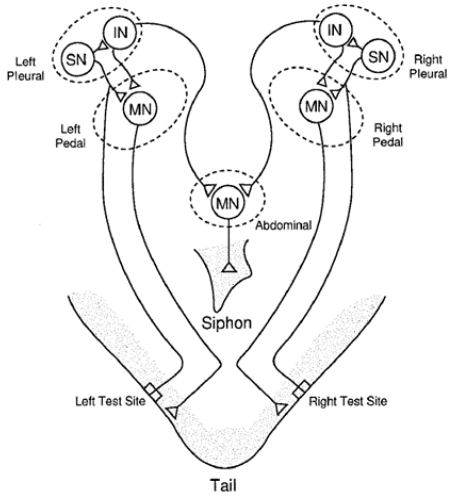


Figure 5 *Schematic image of neural circuitry involving TSWR (Cleary et al., 1998) (SN = sensory neuron; IN= interneuron; MN= motor neuron)*

The network circuitry of the TSNs include making monosynaptic connections onto the motor neurons in the pedal ganglion which instigates tail contraction and the polysynaptic connections onto the motor neurons in the abdominal ganglion which initiates TSWR (Cleary et al., 1995). Figure 6 shows *Aplysia* at 1) a relaxed state, followed by 2) a withdrawn state indicative of the TSWR.

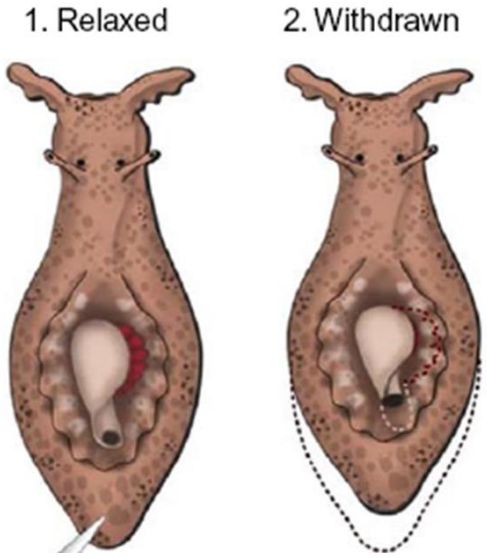


Figure 6 TSWR (Fioravante et al., 2008)

The enhanced TSWR behavior resulting from sensitization training can be translated into its cellular correlates as an increased excitability of the TSNs (Cleary et al., 1998).

The ability to study the association of the defensive behavior and its cellular correlates as well as investigating the non-defensive behavior and its cellular correlates is a unique approach that was taken in this study. It offers a versatile perspective on the learning displayed by the animal when it is subjected to an aversive stimulus.

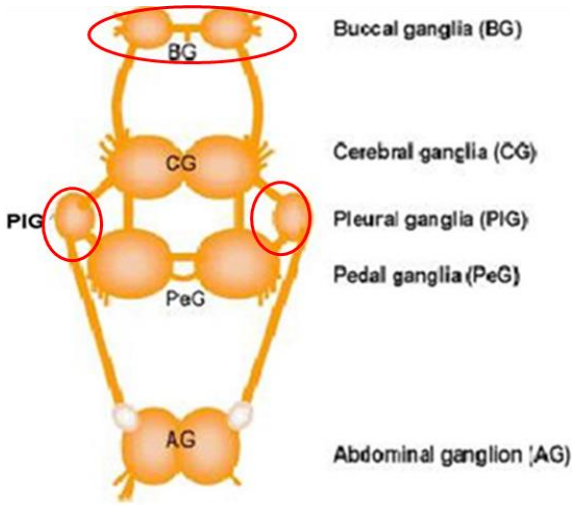


Figure 7 *Aplysia* nervous system indicating pleural and buccal ganglia (asnailodyssey.com)

In vitro Analog

The ability to study the neural correlates associated with specific behaviors is a critical advancement and fundamental concept in understanding behavior. By being able to study the nervous system *in vitro*, neuroscientists have made significant advancements in explaining cellular mechanisms and neural constituents that modulate certain behaviors (Hawkins et al., 1983). Often termed as *in vitro* analogs (Walters and Byrne, 1983), these preparations allow investigations into the molecular mechanisms involved in learning and memory (Hawkins et al., 1983).

In *Aplysia*, there have been developments of *in vitro* analogs to understand the various mechanisms of learning, such as sensitization (Zhang et al., 1994; Acheampong et al., 2012; Weisz et al., 2017). Aversive stimuli can be delivered to the ganglia in the form of a single-training protocol consisting of electrical shocks delivered to identified

peripheral nerves (Weisz et al., 2017). For this project, a reduced preparation of the *Aplysia* ganglionic nervous system consisting of the buccal, cerebral and pleural-pedal ganglia was used (Figure 8). The buccal ganglia contain neurons B51 modulating feeding and the pleural ganglia contain the TSNs, responsible for TSWR (Weisz et al., 2017). Nerves P8 and P9 were also excised as they innervate the body wall through which sensitizing stimuli is delivered (Zhang et al., 1994; Weisz et al., 2017 update).

Previous studies have indicated that when the ganglia are exposed to sensitization training, two distinctive changes occur: 1) there is an increased excitability of TSNs and 2) there is a decreased excitability of the decision-making neuron, B51 (Weisz et al., 2017). These *in vitro* changes correlate with the changes that occur *in vivo*: exposure to sensitization training lead to an enhancement of the TSWR and a decrease in the feeding behavior.

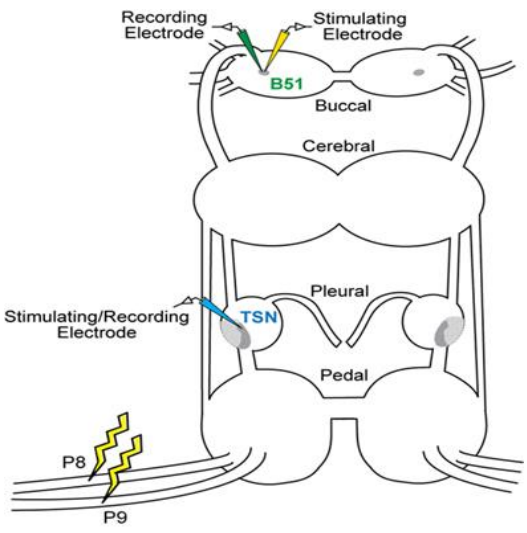


Figure 8 Reduced preparation of the *Aplysia* ganglionic system (image provided by Mozzachiodi Lab)

The Cyclic Guanosine Monophosphate (cGMP) Pathway

The Cyclic Guanosine Monophosphate (cGMP) pathway dependent on protein kinase (PKG) (see Figure 9) is involved in these learning-dependent behavioral and neuronal plasticity that are associated with these defensive and non-defensive responses in *Aplysia* (Michel et al., 2011). Specifically, the cGMP pathway dependent on the PKG

is involved in long-term sensitization in behavioral and neuronal plasticity (Lewin, 1999). To identify the specific molecule involved in these behavioral and neuronal changes at a short-term time point the likely downstream target of this pathway (i.e. PKG) was explored. Therefore, the specific aim of this project was to examine the role of PKG activity on the behavioral and cellular changes induced by sensitization training. To selectively examine PKG, a pharmacological blocker, KT5823 was used to block PKG. This blocker was used as it has identifiable effects on the neural circuits involved in defensive and non-defensive responses, both behaviorally and at the cellular level (Michel et al., 2011). The objectives investigated for this thesis are outlined below:

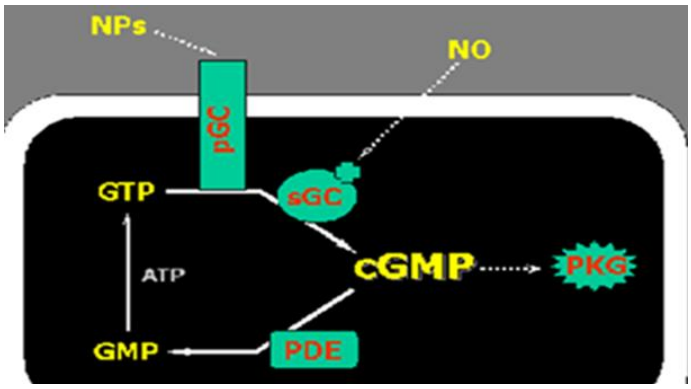


Figure 9 Neuronal pathway (blauplanet.com)

Objective 1: Examine whether the selective PKG inhibitor, KT5823, prevents training-induced suppression of feeding

The role of KT5823 on a non-defensive behavior (feeding) was analyzed by blocking this pathway and determining the effects on the training-induced suppression of feeding. This experiment was conducted *in vivo*.

H_{null}: KT5823 will not influence the training-induced suppression of feeding

$H_{alternative}$: KT5823 will prevent training-induced suppression of feeding

Objective 2: Examine whether the selective PKG inhibitor will affect the expression of sensitization of the TSWR

The role of PKG was assessed in the expression of sensitization involving TSWR. It was analyzed whether blocking this pathway with KT5823 will influence the training-induced expression of sensitization. The defensive response, TSWR, was elicited by delivering electrical stimuli (20-ms pulses) via implanted electrodes. This experiment was conducted *in vivo*.

H_{null}: KT5823 will not influence the expression of sensitization

$H_{alternative}$: KT5823 will influence the expression of sensitization

Objective 3: Examine whether KT5823, PKG inhibitor, prevents decreased B51 excitability and increased TSN excitability

This objective assessed the role of PKG in the cellular correlates, specifically in TSNs and neuron B51. It was analyzed *in vitro* whether PKG activity is required for the increased excitability in TSN and decreased excitability in neuron B51 induced by training.

H_{null}: KT5823 will not have an effect on the decreased B51 excitability and increased TSN excitability

$H_{alternative}$: KT5823 will prevent training-induced decreased B51 excitability and increased TSN excitability

MATERIALS AND METHODS

Location and Logistics

All experiments were conducted within the Mozzachiodi Neuroscience laboratory at Texas A&M University, Corpus Christi. Experimental protocols had approved by the institution and therefore permission to execute experiments had been granted.

Animal Maintenance

Aplysia were collected from the Southern California coast by open water divers employed by South Coast Bio- Marine LLC. Once animals arrived, they were kept in aquaria that were housed in the tank room adjacent to the lab. The tank room comprised of two aquaria of 530 L each of artificially made seawater. Tank water was continuously circulated and regularly monitored at 15ºC, mimicking conditions from which the animals were collected. This seawater was made using Instant Ocean™ to maintain salinity levels at 34-36 ppm. The tank room was set to a diurnal light and sleep cycle where animals received 12-hr of light and 12-hr of darkness. Furthermore, each animal was individually housed within a rectangular plastic container that was perforated to allow for water circulation (figure 10).

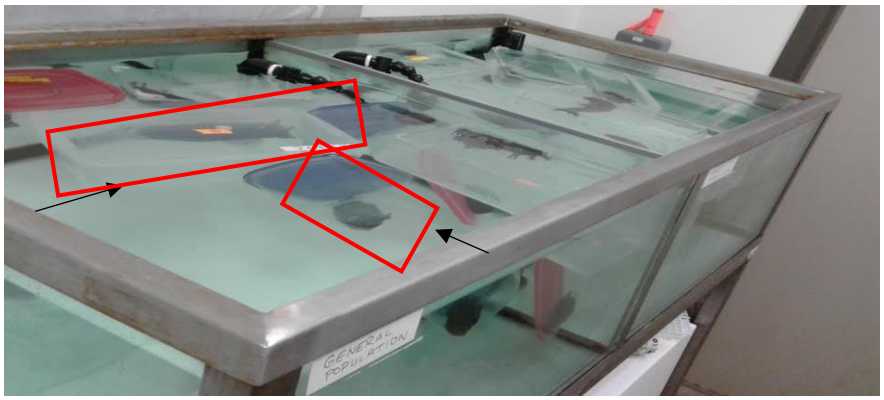


Figure 10 Image of tank room-arrows indicate examples of cages housing individual animals

Upon arrival, an acclimation period of three days was implemented before animals were used in experiments (Levenson et al., 1999). Animals were fed one strip of red seaweed (Emerald Cove Organic Pacific Nori) three times a week on Monday, Wednesday, and Saturday (figure 11). If experiment days were altered, then the feeding schedule was adjusted accordingly to ensure animals were always 48-hr food deprived

before being used for an experiment. Furthermore, if an animal laid eggs, and/or secreted ink and/opaline, then the animal was not used for a minimum of seven days.

Figure 11 *Aplysia* ready to ingest a piece of seaweed

Experimental Design

Objective 1: Examine whether the selective PKG inhibitor, KT5823, prevents training-induced suppression of feeding

Seaweed extract preparation

To induce bites (figure 12) in the animal, a seaweed extract (SWE) was used (Brembs et al., 2002; Acheampong et al., 2012). The SWE was prepared the day of the experiment to ensure freshness of the food stimulus. Organic Pacific Nori dried seaweed as used to create the seaweed extract. A half sheet, weighing ~ 1.25 g, was cut into small squares and then immersed in a 300mL flask of tank water. Next, this solution was mixed for a total of 30-min to allow the seaweed pieces to disintegrate. After the 30-min, the seaweed extract was filtered into a container using a coffee filter. The final extract was then stored in a 15ºC refrigerator until it was needed for experiment use (Acheampong et al., 2012).

Figure 12 *Aplysia* 'making a bite'

Feeding Behavior- Biting Test

Feeding behavior was measured by presenting the animal to SWE. Upon contact to the SWE, animals would elicit bites (Figure 12) that were then used to measure and quantify the non-defensive behavior. A pedestal bowl was used to pour 167mL of SWE to 1333mL of artificial sea water (ASW). This accounted for a total of 1500mL of SWE solution. Prior to immersing the animal in the bowl, materials including camera, stopwatch, and datasheets were set up to record data. The animal was transferred from the original container to the bowl using a strainer. Care was taken to not startle the animal during transfer because doing so may have triggered ink and/or opaline release. In the event the animal would release any ink and/or opaline, the animal was terminated from the experiment as it would run the risk of already being sensitized (Cleary et al., 1998; Acheampong et al., 2012).

As soon as the animal was released into the bowl, it was noted when the animal attached to the surface of the bowl. Once the animal attached, the timer was started to account for a 5-min period, and the number of bites was counted. One complete bite included the opening of the mouth, protruding of the radula (Kupfermann, 1974), and then retracting of the mouth as demonstrated in Figure 13. In order to establish a baseline for comparison of number of bites post training, a minimum of 5 bites was required during the 5-min pre-test (Acheampong et al. 2012). As soon as the 5-min interval was completed, the animal was placed back into its original container.

Figure 13 Protraction and retraction (Nargeot and Simmers, 2011)

The first feeding behavior test was determined to be the "pre-test" which served as a baseline to compare the number of bites the animal produced. The same protocol was implemented for the 15-min and 2-hr posttests experiments. Feeding tests were recorded to allow for re-counts in case bites were missed.

Injection Procedure

To inhibit PKG signaling, experimental animals were injected with the inhibitor, a pharmacological drug, KT5823. Previous work by Michel et al. (2011), indicated that using the drug KT5823 at a 6.5 µM/100 g concentration induced operant memory in *Aplysia*. Animals were injected with a concentration of 6.5 µM/100 g weight of KT5823 mixed with normal sea water (Michel et al., 2011). Control animals were injected with a concentration of stock solution of normal sea water and dimethylsulfoxide (DMSO). For every 100 grams of body weight, a 6.5 µM of this stock solution was injected into the animal (Michel et al., 2011).

Injections were applied into the body wall, angling towards the rhinophore (Figure 14). In the event the animal found the injection aversive, it would secrete ink and/or opaline. In this case, the animal was terminated from the experiment and euthanized. If the injection treatment was not aversive to the animal, then the animal was indicated to have 'passed' the pre-test and able to be used in the experiment.

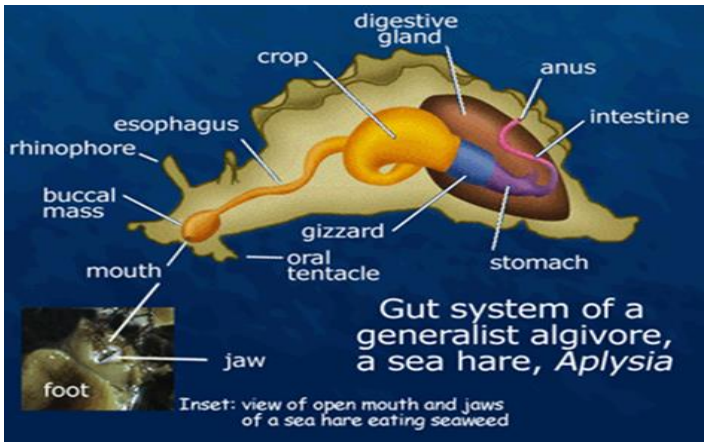


Figure 14 Gut system of *Aplysia* (lanwebs.lander.edu)

Training procedures

An hour post injection, the animal was assigned to either a 'trained' or 'untrained' treatment group. The experimenter remained blind to the animal's training history until after the experiment was concluded to avoid bias. If the animal was 'trained', it would receive 10 shocks (500 ms, 10 Hz, 60 MA AC) in the form of electrical stimuli to the body wall of the animal to stimulate an aversive response (Scholz and Byrne, 1987; Cleary et al., 1998). When subjected to the aversive stimuli producing sensitization, there is an induced suppression of the feeding behavior and an enhancement of the TSWR that occurs at the short-term time period: 15-min and 2-hr post training (Cleary et al., 1988; Acheampong et al., 2012; Shields-Johnson et al., 2013). This aversive response was determined to have occurred if the animal released opaline and/or ink. Animals that were untrained received identical treatment in terms of handling, but did not receive electrical stimuli to the body wall (Acheampong et al., 2012).

The first post-training test was done at 15-min after the treatment was applied. The same procedure for conducting the feeding behavior "pre-test" as mentioned above was applied for conducting the feeding behavior 15-minute post-test. After this second

behavioral test was completed, the animal was returned to its original experimental bowl. Next, 2-hr post injection time, a third and final behavioral test was conducted.

After the third feeding behavioral test was completed, the snail was euthanized with isotonic magnesium chloride solution that was stored in a 15ºC cooling refrigerator. The amount of magnesium chloride that was injected equaled that of 50% of the body weight of the animal (Mozzachiodi et al., 2003). After euthanizing, the animal was placed into a zip-lock bag in the freezer for disposal (Wolfe et al., 2016).

For this experiment, four different training/treatment groups were used: trained/injected with KT5823, untrained/injected with KT5823, trained/injected with vehicle solution, and finally, untrained/injected with vehicle solution. Details of statistical analyses conducted are explained below.

Objective 2: Examined whether the selective PKG inhibitor affected the expression of sensitization

To study the expression of sensitization, the defensive behavior- tail siphon withdrawal reflex (TSWR), was examined in the animal. Prior to conducting the TSWR experiment, two procedures were executed: the parapodectomy and the electrode implantation. The parapodectomy was executed to ameliorate the view of the siphon while electrode implantation was necessary to allow transfer of electrical current.

Protocol for Parapodectomy

One week prior to the experiment, the animal underwent a surgical procedure, called a parapodectomy, where a part of the parapodia was removed. Since measuring the TSWR requires a complete view of the siphon to allow accuracy of contraction and relaxation measurements, the parapodia were trimmed off. The parapodia (figure 15) is a structure on the animal that encloses the siphon.

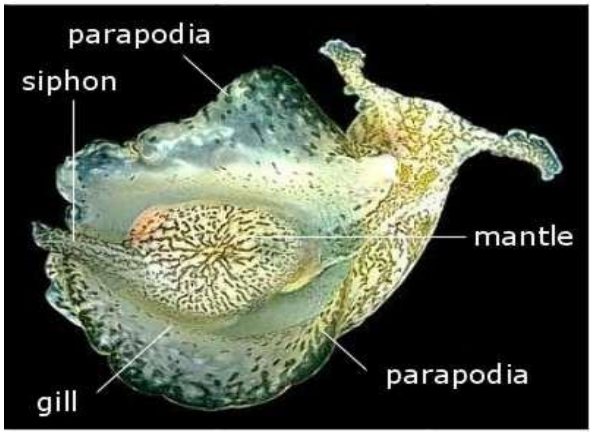


Figure 15 *Aplysia* anatomy (Seaslugforum.net)

The parapodectomy was done after keeping the animal under ice for a total of 18-min, thereby anesthetizing it. Forceps were then used to gently touch the rhinophore and siphon to ensure that the animal was not awake. In the event that the animal was not fully anesthetized, the animal was given about two minutes extra underneath the ice. Once the animal was completely anesthetized, a hemostat was taken and clasped onto the posterior part of the left parapodium. Next, while holding the hemostat clamped onto the parapodium, a scissor was used to trim off the excess of the top part of the parapodium (figure 16). After this was completed, the hemostat remained clasped onto the parapodium for about 30 s to allow the tissue to heal.

Figure 16 Hemostat used to clamp parapodium

During this period, the exact procedure was repeated on the right side of the parapodium using another hemostat. After 30 s, the hemostat was released, and the animal was gently placed back into a bowl of artificial sea water. Care was taken to conduct the surgery swiftly as once out of the ice, the animal began the process of awakening. The animal was given time to 'wake up' before being placed back into the tanks in the tank room. At any point of the parapodectomy, or after the surgery, if the animal secreted ink and/or opaline, then the animal was not used in the experiment.

Protocol for Implanting Electrodes

The electrical stimulation necessary to elicit the TSWR was delivered through Teflon-coated silver wires implanted into the animal's tail. Wires were cut to a length of six inches and one end of the wire was burnt briefly to have the end exposed. The exposed ends which were about 0.5 cm in length, was then inserted into a needle that was attached to a syringe. After insertion, the wire was coiled six times around the needle to ensure that the wire did not fall off the needle. Once needles were ready for insertion, the animal then underwent the process of anesthetization, which included being immersed in ice for 18-min (Goldsmith and Byrne, 1993; Acheampong et al., 2012).

Once animals were fully anesthetized, two wires were implanted on either the left or right side of the animal's tail (figure 17). The side of the tail was typically chosen at random; in the event of an unusual circumstance, such as a deformed tail, then the side with a larger surface area was chosen instead. The wires were then inserted one after the other into the tail of the animal. After successful wire implantation, animals were placed back into the artificial sea water to allow time to revitalize.

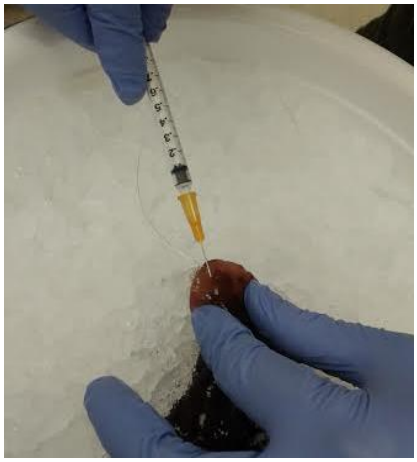
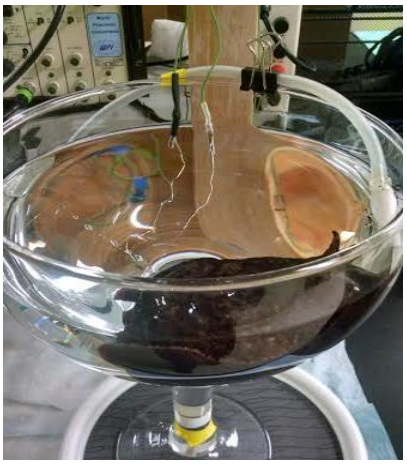
A  **B** 

Figure 17 **A** Implanting wires into the tail **B** Animal hooked up with two implanted electrodes

This process was conducted three days before the experiment was executed to allow the animal to recover from the electrode implantation procedure. If the animal lost any electrode during this time point, then the animal was eliminated from the experiment. Finally, the animals were placed in a side of the tank that was isolated to be "food-deprived" and were therefore on their own feeding schedule. One day after electrode implantation, the animal was fed a strip of seaweed. This was then followed by a 48-hr food deprivation period (as the feeding protocol entailed) before the TSWR experiment was conducted.

TSWR Measurement Protocol

In order to determine the minimum amount of current required to induce the TSWR, electrical stimuli (20-ms pulses) was delivered via the implanted electrodes in 0.2mA increments (Cleary et al., 1998; Wainwright et al., 2002). The electrical stimulus that instigated the TSWR response was determined as the "threshold stimulus" for the animal. Once the threshold stimulus was obtained, the animal was given a 30-minute period to acclimate post threshold stimulus testing. During this time period, 400 mL of 4°C tank water was exchanged with the water from the experimental bowl. This was done to maintain the temperature at 15 °C, to limit any behavioral changes that may occur from temperature fluctuations (Cleary et al., 1998).

Next, the particular threshold stimulus that was obtained was doubled so that this threshold intensity would reliably instigate a response from the animal (Goldsmith and Byrne, 1993; Cleary et al., 1998; Wainwright et al. 2002). Once the 30-minute interval was over, the initial TSWR testing (i.e. pre-test) consisted of five test stimuli that were delivered at 10-min apart. Each time the animal received the stimulus, the duration of siphon contraction and relaxation was recorded (Figure 18). Once all five tests were conducted, an average was taken to determine the TSWR duration for the pre-test value. In the event the average TSWR duration exceeded 10 s, then the animal was excluded from this experiment as the animal may have already been sensitized and therefore may give inaccurate results (Wainwright et al., 2002).

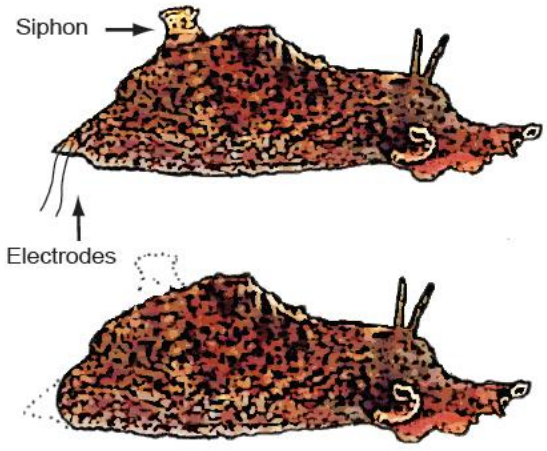


Figure 18 Illustration of siphon contract and relaxation

Once this pre-test average had been determined, the animals were transferred from the experimental bowl to another bowl where they were subjected to a specific treatment. Identical injection procedures were followed as outlined in objective 1 where experimental animals were injected with KT5823 while control animals were injected with the solution of the normal ASW and DMSO mixture. One-hour post injection, animals were either trained via a single-trial sensitization training (10-s 1-Hz train

comprised of 500-ms pulses) or were not trained (Acheampong et al., 2012) (see Figure 19 for training and treatment visual).

The TSWR testing was then repeated 15-min and 2-hr after training (Acheampong et al., 2012). This requirement of testing exactly at the 15-min and 2-hr time mark did not allow for an opportunity to take an average as was done in the pre-test and so only one TSWR testing was point was determined for each post-test. After the animal had completed the pre- and post-tests, the two mean TSWR durations were then calculated as a ratio (post/pre). This TSWR ratio was then used as a measure of sensitization to compare between the four groups. Once the testing was over, animals were euthanized as outlined in objective 1.

Figure 19 **A** Experimental Set-Up. **B** Image of animal receiving electrical shock

For this experiment, four different training/treatment groups were used: trained/injected with KT5823, untrained/injected with KT5823, trained/injected with vehicle solution, and finally, untrained/injected with vehicle solution. Details of statistical analyses conducted are explained below.

Objective 3: Examined the role of PKG activity in TSN excitability increase and B51 excitability decrease after *in vitro* training

To assess the involvement of PKG activity at a cellular level, an *in vitro* analysis was conducted in conjunction to the behavioral experiments. This was done to allow for a parallel perspective of comparing behavioral results to their cellular correlates. Neurons controlling the feeding and the TSWR circuitry were therefore examined for this project.

Specifically, the activities of neuron B51- controlling the feeding behavior, and the tail sensory neurons (TSN) controlling the TSWR, were assessed.

Once a 48-hr food deprived animal was selected from the tank room, a piece of seaweed was presented to the animal to trigger a bite. This indicated that the animal was in a healthy, motivational state and could thereby move forward for *in vitro* analysis as the feeding circuit remained functional. As soon as it passed the bite test, the animal was weighed and anesthetized by injecting isotonic $MgCl_2$ (the volume equaled to 50% of the body weight) through its foot into the hemocoel. After waiting several minutes, forceps were used to touch the rhinophores to assess if the animal was responsive to the tactile stimulus. If the animal was not responsive, then this confirmed that the animal was anesthetized.

Removal of the Buccal and Pleural-Pedal Ganglia

The ganglia containing the buccal and pleural-pedal ganglia (Figure 20) were excised from the animal. From the buccal ganglion, the third branch of the peripheral buccal nerves, (Bn.2, 3) were removed. The Bn. 2,3 nerve was useful to have to help identify neuron B51 as the nerve stimulation can induce feeding behavior in the form of B51 firing patterns (Shields-Johnson et al. 2013; Dickinson et al. 2015; Weisz et al., 2017). In addition, nerves P8 and P9, attached to the pleural-pedal mass, were also removed. This was done because nerve P9 is required to identify tail sensory neurons (TSNs) which account for the TSWR that was measured behaviorally (Weisz et al., 2017) (as outlined in Objective 2). In order to create the parallel training protocol from the *in vivo* paradigm to the *in vitro* paradigm, the nerve P8 was also required. This electrical stimulation to nerves P8/P9 was analogous to the behavioral training which resulted in

feeding suppression and sensitization at least 15-min after the stimulation was delivered (Acheampong et al., 2012). The *in vitro* experiments further established that electrical stimulation via nerves P8/P9 induced sensitization training and so were used for this particular experiment (Weisz et al., 2017).

After the ganglion and the respective nerves were removed, they were transferred to a petri dish that contained 1500 µL of Hi-Di solution. This solution contained artificial seawater (ASW) and a high concentration of Ca^{2+} and Mg^{2+} cations. The purpose of the Hi-Di ASW was used to temporarily inhibit neural activity while the ganglia were pinned down to the dish and desheathed (Byrne et al., 1978 Nargeot et al., 1997).

Once the buccal ganglia and the peripheral nerves consisting of nerves P8 and P9 were removed and pinned to a Sylgard-coated dish, a Vaseline well was constructed on the periphery of the dish. This 'well' served to isolate nerves P8 and P9 from the saline contained within the dish as extracellular electrodes were placed in these nerves. The nerves, P8 and P9, was stimulated by sensitizing stimuli which serves to mimic the induction of sensitization (Zhang et al., 1994; Weisz et al., 2017).

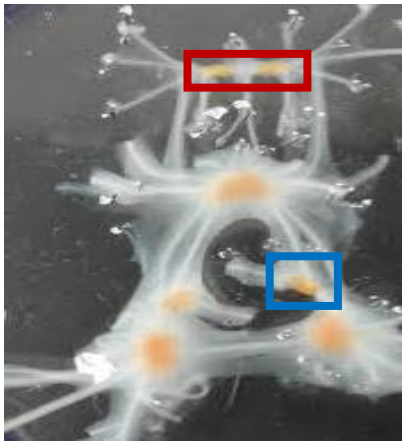


Figure 20 *Aplysia* ganglia consisting of desheathed buccal ganglia (red outline) and sensory cluster (blue outline)

The next step was to desheath the buccal and pleural masses which required the connective tissue covering the masses to be removed. This was done by taking forceps and

scissors to carefully cut away the tissue covering, thereby exposing the cells to be impaled. Once the buccal and pleural ganglia were desheathed, and the extracellular electrodes were placed so as to be in contact with nerves P8 and P9, the Hi-Di ASW was then exchanged for normal ASW. This was done by taking out 500 µL of HI-Di and then replacing with 500 µL of normal ASW for a total of 15 times to have a final solution volume of 1500 µL in the dish. Furthermore, a period of 30-min was allotted to ensure that the entire nervous system regained synaptic activity before measurements were taken (Shields-Johnson et al., 2013). During this time, the temperature of the bath within the petri dish was kept at 15°C using a temperature controller. Again, this mimicked the temperature the animal typically lives in thus allowing cellular conditions to be similar to behavioral conditions.

Also during the 30-minute duration, glass electrodes were created using a Micropipette puller (figure 21).

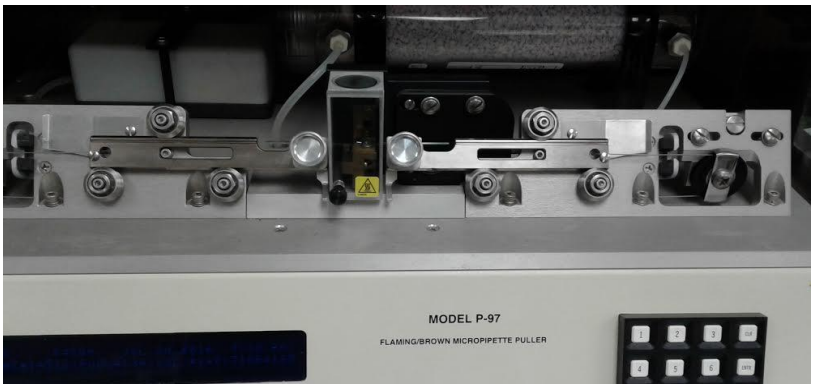


Figure 21 Image of micropette puller machine

After the electrodes were created, they were filled with potassium acetate and allowed to sit to let out any air bubbles. Once the 30- minute time period was over, two glass microelectrodes were then used to impale neuron B51 of the buccal ganglion (Figure 22). A standard two-electrode current clamp technique was implemented to obtain the intracellular recording and measurement of B51 activity. Since clamping B51 often required adjusting for large current changes, this method was beneficial as one microelectrode was used to inject current and the second one was used to account for the

voltage readings (Nargeot et al., 1999). A third microelectrode was used to impale a tail sensory neuron (TSN) in the plural ganglia (Zhang et al., 1994). TSNs are relatively smaller neurons and therefore were only impaled with one electrode to prevent cell damage (Weisz et al., 2017). After the three electrodes were successfully implanted in the respective neurons, a resting period of five min were allowed for the cells to recover from the impalement.

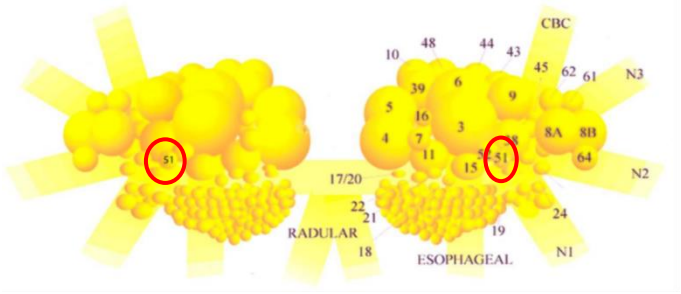


Figure 22 Neurons B51 in red circle (image provided by Dr. Riccardo Mozzachiodi)

In vitro measurements

The measurements that were taken *in vitro* followed a training protocol depicted below (Figure 23). Properties of B51 and TSN were taken as pre-test measurements and then treatment was bath applied. Ten minutes post treatment, the ganglia either received the sensitization training or did not. After a period of 15-min, post-test measurements of B51 and TSN were taken.

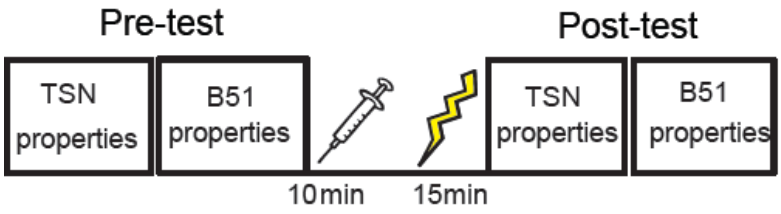


Figure 23 *In vitro* testing and training protocol illustration

The first measurement that was taken for neuron B51 was the resting membrane potential. Once the resting membrane potential was obtained, the cell was then clamped at

-60 mV. After clamping, the input resistance was then measured by subjecting the cell to a 5 s, 5 nA of hyperpolarizing current. Ohm's Law (I=V/R) was then used to calculate input resistance by taking the voltage difference and dividing by the current (5nA).

The third measurement that was taken was the burst threshold (Bt) of B51. B51 exhibits a unique firing pattern which is a characteristic burst of action potentials called plateau potentials (Figure 24) (Nargeot et al., 1999).

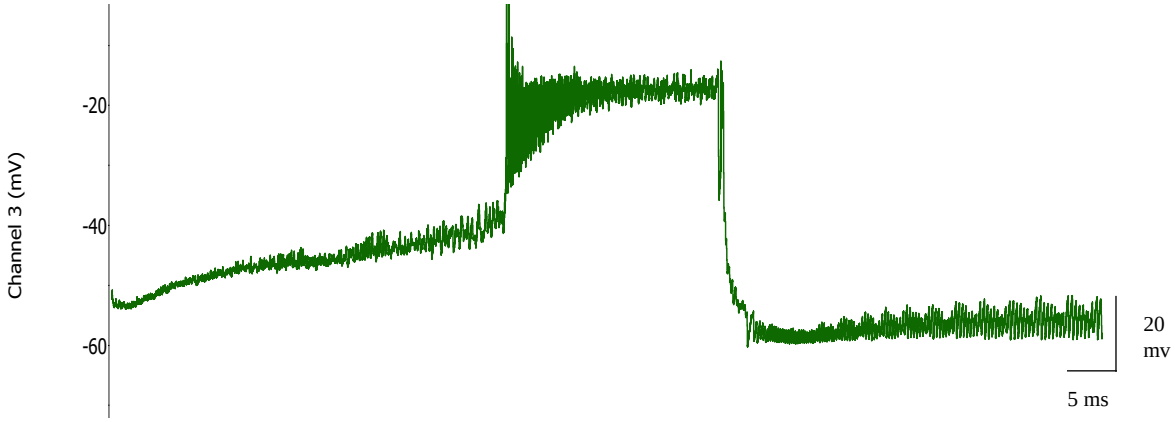


Figure 24 Spontaneous plateau exhibited by neuron B51 with electrodes

The threshold needed to elicit a plateau potential in B51, known as the burst threshold, was then measured by stimulating the cell with depolarizing current of 5 s pulses beginning at 5 nA. This depolarizing current was increased incrementally by 1 nA until the cell fired a plateau potential. Care was taken to ensure B51 was always clamped at -60 mV throughout all measurements to allow for a comparative baseline between all measurements. Once the measurements were taken, B51 was unclamped until the next part of the experiment. (Nargeot et al., 1999) (Figure 25).

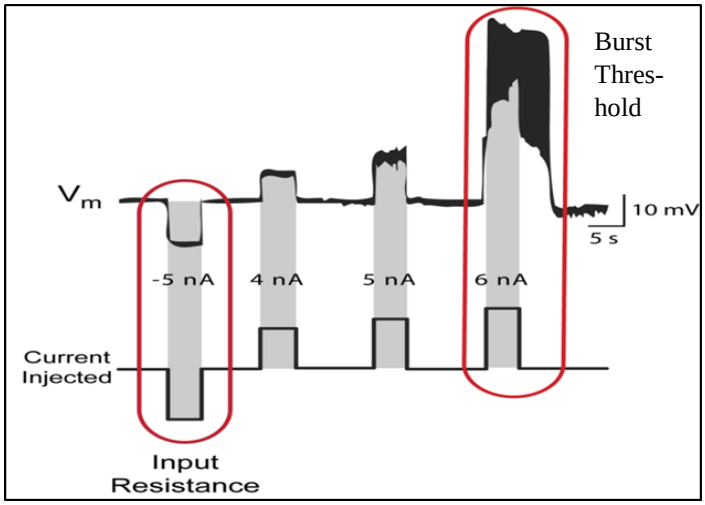


Figure 25 Example of measurable properties of B51 (Dr. Riccardo Mozzachiodi)

Similar to initial B51 measurements, the resting membrane potential of a tail sensory neuron was also determined and then current clamped to -45 mV (Baxter and Byrne, 1990). In order to measure the excitability of the TSN, the number of action potentials (Figure 26) generated by the application of a 1 s of 2 nA depolarizing current was measured (Cleary et al., 1998).

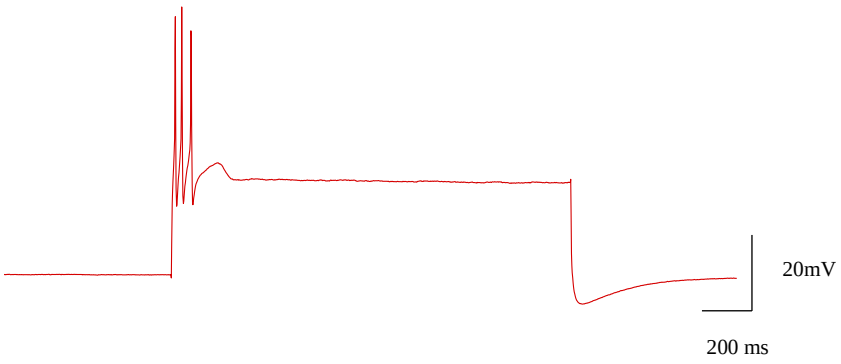


Figure 26 Example of TSN spikes

The membrane properties of both B51 and TSN neurons were taken prior to (pre-test) and 15-min after (post-test) training. Ten minutes after the pre-test measurements were taken, the treatment solution was applied to the bath. Half of the preparations had the control solution (vehicle-2 µM) applied to the bath, while the remaining preparations

had the pharmacological blocker (KT5823- 2μM) applied to the bath. Of those preparations, half were randomly selected to be trained and the remaining were untrained.

This training duration consisted of a single 10-s block of 10, 500-ms trains. The training was comprised of 3-ms pulses at a frequency of 60 Hz and intensity of 60 mV which stimulate the nerves P8 and P9 (Weisz et al., 2017). The ratio excitability of the pre-test and post-test were determined for B51 and TSN neurons respectively. These measurement protocols were identically followed for both trained and untrained preparations. Untrained preparations served as the control and did not receive the electrical stimuli. In total, there were four treatment groups: trained/KT5823, untrained/ KT5823, trained/vehicle, and untrained/vehicle. Details of statistical analyses conducted are explained below.

Statistical Analyses

Behavioral and cellular data were computed using previously-established procedures. Differences in post-test and pre-test results for the feeding behavioral experiments were determined as "post-test – pre-test" (e.g., Lechner et al., 2000; Lorenzetti et al., 2006; Acheampong et al., 2012; Wolfe et al., 2006). For the TSWR behavioral experiments ratios were determined as "post-test/pre-test" (e.g., Scholz and Byrne, 1987; Goldsmith and Byrne, 1993; Acheampong et al., 2012). These calculations were determined at both the 15-min and 2-hr time points for both feeding and TSWR behavioral experiments. For *in vitro* results, percent changes were computed by using the formula [(post-test-pre-test)/pre-test]X100 (Nargeot et al., 1999; Mozzachiodi et al., 2008; Weisz et al., 2017). The post-test time point for this experiment was 15-min (Weisz

et al., 2017). In all experiments, these results were compared among the four treatment groups: trained/KT5823, untrained/ KT5823, trained/vehicle, and untrained/vehicle

Statistical analyses and respective illustrations were created using the software SigmaPlot. For all statistical analyses, the statistical significance was set at $p<0.05$ and illustrated as mean ± SEM. All experiments were analyzed using the Kruskal-Wallis test (*H*) with the non-parametric Student-Newman Keuls (*q*) (SNK) method for post hoc pairwise analysis (Mozzachiodi et al., 2003; Mozzachiodi et al., 2008; Acheampong et al., 2012). In a situation where two sets of data were compared, the Mann-Whitney test was used for analysis (Mozzachiodi 2017, personal communication). Statistical results were then illustrated in the form of vertical bar graphs that were also created using the SigmaPlot software.

RESULTS

Part IA – Effects of KT5823 on training-induced suppression of feeding

The first aim of the project assessed the role of the selective PKG inhibitor, KT5823, on the training-induced suppression of feeding. Results indicated that at the 15-min time point there was a statistically significant difference ($H=15.13$, $P=0.002$) among the four groups (n=18 for each group): vehicle/untrained, vehicle/trained, KT5823/untrained, and KT5823/trained (Figure 27). The SNK test determined significant differences in groups: vehicle/untrained vs vehicle/trained ($q=4.758$; $p<0.05$), vehicle/untrained versus KT5823/trained ($q=5.146$; $p<0.05$), KT5823/untrained versus vehicle/trained ($q=4.832$; $p<0.05$) and KT5823/untrained versus KT5823/trained ($q=5.448$; $p<0.05$). Based on these results and the lack of significance between and

among the other groups suggested that KT5823 did not block the suppression of feeding at the 15-min time point.

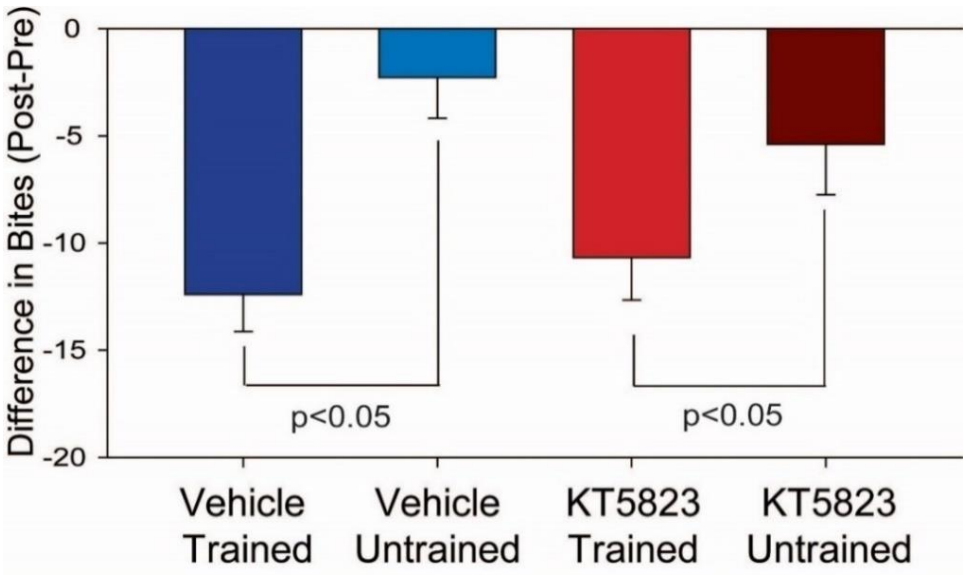


Figure 27 Role of KT5823 (6.5μM/100g) in suppression of feeding at the 15-min time point

At the 2-hr time point a similar trend was found when compared to the 15-min results. Results indicated that there was not a statistical significant difference ($H = 3.543$ with 3 degrees of freedom; $p = 0.315$) among the four groups (n=18 in all groups): vehicle/untrained, vehicle/trained, KT5823/untrained, and KT5823/trained (Figure 28). The plausible reason for lack of significance is probably due to the drop in the number of bites depicted by KT5823/untrained animals. At the 2-hr time point, this drop is perhaps a consequence of drug injection into the animal. Therefore, these differences of the mean values suggest the differences may simply arise from the sampling variability itself.

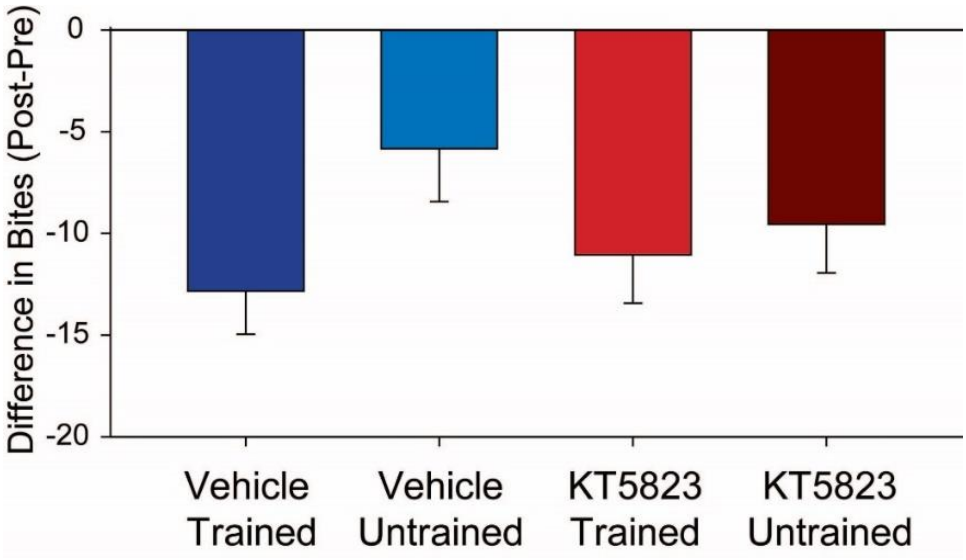


Figure 28 Role of KT5823 (6.5µM/100g) in suppression of feeding at the 2-hr time point

Part I B- Examining a higher concentration of KT5823 on the training-induced feeding suppression

Prior to analyzing the effect of KT5823 on the training-induced expression of sensitization in the TSWR experiment, I decided to use a higher KT5823 concentration to ensure that the lack of effect on feeding suppression was not due to the use of a sub-threshold drug concentration. An additional experiment was conducted using KT5823 at a doubled concentration (i.e., 13.0 µM/100 grams). Testing, training and KT5823/vehicle preparation and injection were identical to those illustrated in the Material and Methods section for the experiment with 6.5µM/100g. Comparison using the Mann-Whitney test indicated that both vehicle/trained and KT5823/trained groups expressed feeding suppression at the 15-min ($p=0.741$) and at the 2-hr time points ($p=0.230$), indicating that the doubled concentration did not block learning.

Part II – Effects of KT5823 on training-induced expression of sensitization

The second project examined whether the selective PKG inhibitor (KT5823 at 6.5 µM/100g) affected the expression of sensitization in *Aplysia*. Statistical analysis revealed a significant difference ($H = 21.492$ with 3 degrees of freedom; $p = <0.001$) among the

four treatment groups for the 15-min time point (n=12 in all groups). Post-hoc analyses determined differences in groups: vehicle/trained versus KT5823/untrained (q=4.856; p<0.05), vehicle/trained versus vehicle/untrained (q=6.247; p<0.05), KT5823/trained versus KT5823/untrained (q= 6.055; p<0.05) and KT5823/trained versus vehicle/untrained (q= 8.716; p<0.05) (Figure 29).

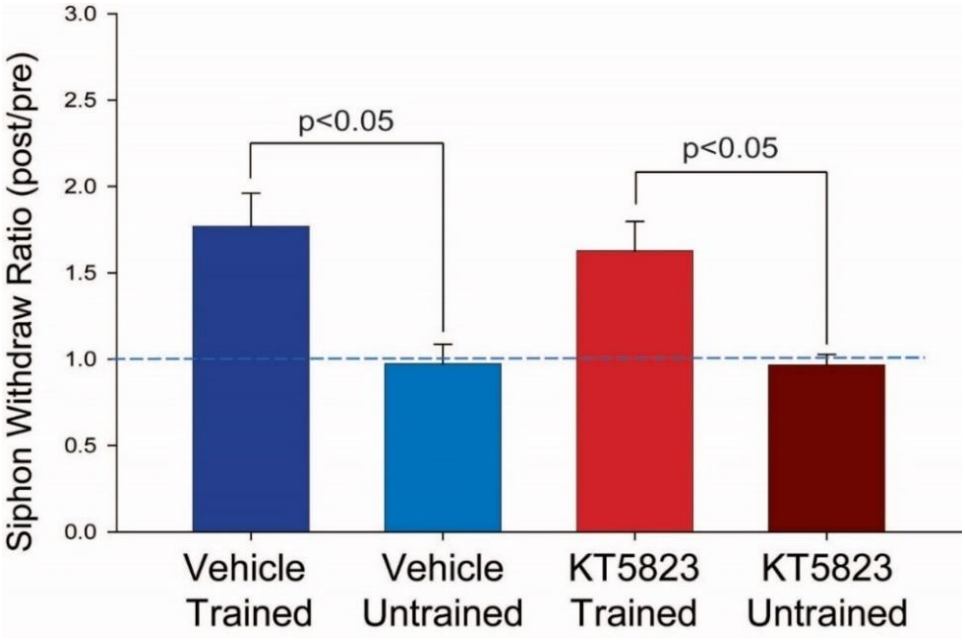


Figure 29 Role of KT5823 (6.5µM/100g) in the expression of sensitization at the 15-min time point (The dotted line indicates the TSWR ratio of 1, and therefore, a ratio greater than 1 indicates that learning in the form of sensitization had occurred)

At the 2-hr time point, a significant difference (H = 19.375 with 3 degrees of freedom; p = <0.001) was found among the four treatment groups (for all groups n=12). Post-hoc analyses determined differences in groups: vehicle/trained versus KT5823/untrained (q=5.576; p<0.05), vehicle/trained versus vehicle/untrained (q=5.103; p<0.05), KT5823/trained versus KT5823/untrained (q=7.042; p<0.05) and KT5823/trained versus vehicle/untrained (q=5.932; p<0.05) (Figure 30).

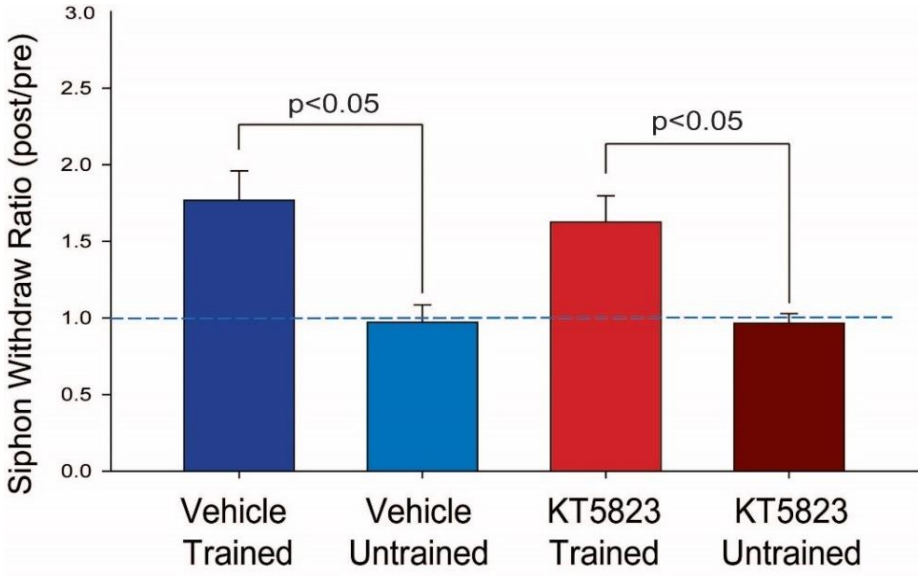


Figure 30 Role of KT5823 (6.5µM/100g) in the expression of sensitization at the 2-hr time point

Part III– Effects of KT5823 on training-induced decreased B51 excitability and increased TSN excitability

The third objective examined whether the selective PKG inhibitor affected training-induced decreased B51 excitability and increased TSN excitability in *Aplysia*.

B51 Excitability

Statistical analysis revealed a significant difference (H=17.566 with 3 degrees of freedom; p= <0.001) for decreased B51 excitability among the four treatment groups 15-min post treatment (n=10 in all groups). Post-hoc analyses determined difference in groups: vehicle/trained versus KT5823/untrained (q=6.017, p<0.05), vehicle/trained versus vehicle/untrained (q=5.396, p<0.05), KT5823/trained versus KT5823/untrained (q=4.196, p<0.05), KT5823/trained versus vehicle/untrained (q=3.969, p<0.05) and vehicle/trained versus KT5823/trained (q=4.757, p<0.05) (Figure 31).

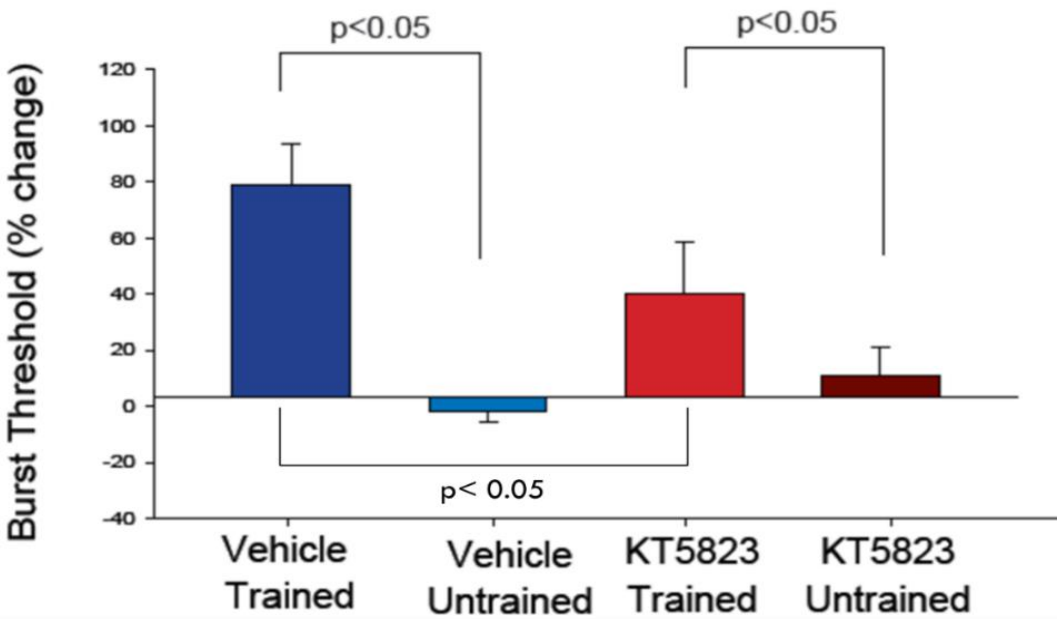


Figure 31 Role of KT5823 in B51 excitability

These changes in burst threshold for the respective groups are illustrated below in the form of traces (Figure 32-35) to depict the effect each bolus (vehicle or KT5823) had on trained and untrained preparations.

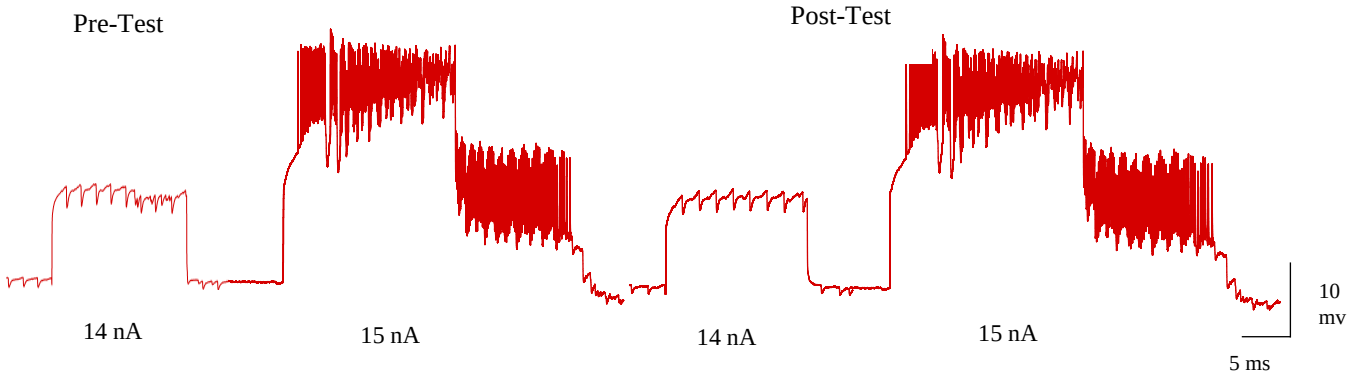


Figure 32 Intracellular recording representative of Vehicle Untrained (Pre-Test and Post-Test respectively) Preparations

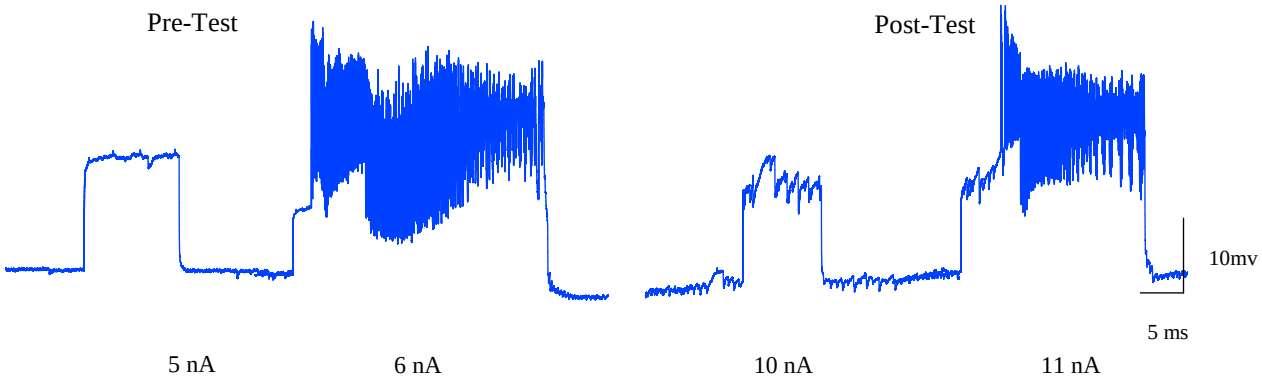


Figure 33 Intracellular recording representative of Vehicle Trained (Pre-Test and Post-Test respectively) Preparations

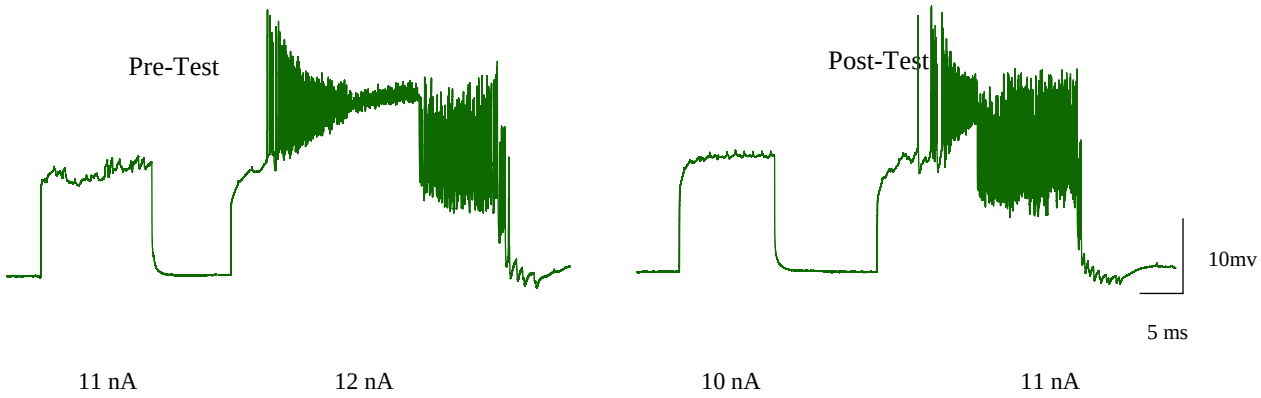


Figure 34 Intracellular recording representative of KT5823 Untrained (Pre-Test and Post-Test respectively) Preparations

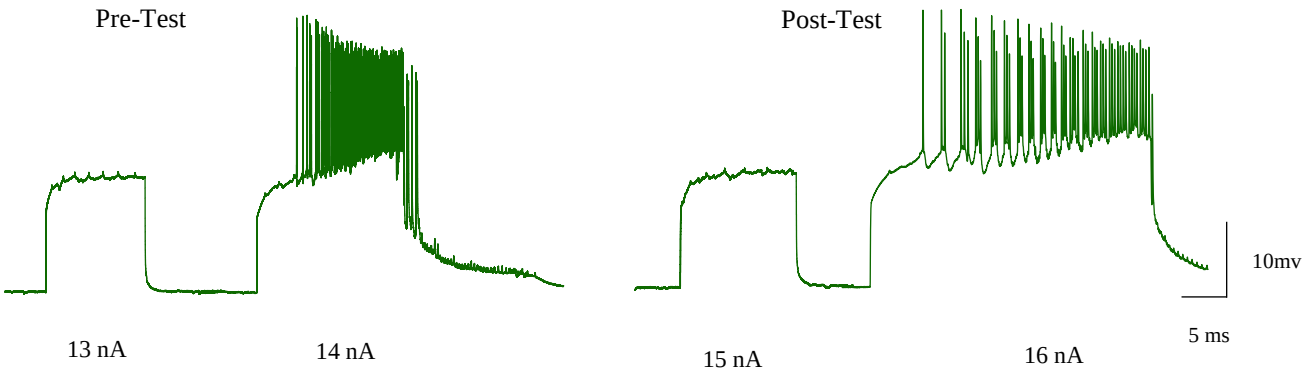


Figure 35 Intracellular recording representative of KT5823 Trained (Pre-Test and Post-Test respectively) Preparations

Additional B51 properties that were measured were resting membrane potential and input resistance (both calculated as a percentage change). The graph below (Figure 36) illustrates percentage change for resting membrane potential of the four groups (n=10 in all groups). Analysis revealed a statistically significant difference (H=10.566 with 3 degrees of freedom; p= 0.014) among four groups. Post-hoc analyses determined: vehicle/trained versus KT5823/untrained (q=3.787; p<0.05) and vehicle/untrained versus KT5823/untrained (q=4.993; p<0.05).

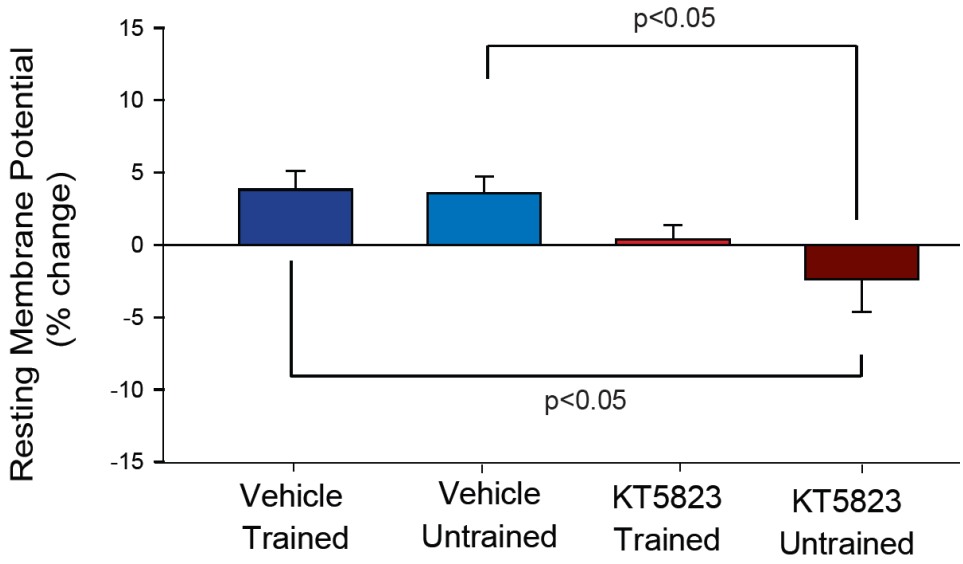


Figure 36 Role of KT5823 in B51 resting membrane potential

The third membrane property that was measured was input resistance (depicted in Figure 37) and was also measured as a percentage change for the four treatment groups 15-min post treatment (n=10 in all groups). There was not a statistical difference among the four groups (H=3.347 with 3 degrees of freedom; P=0.341).

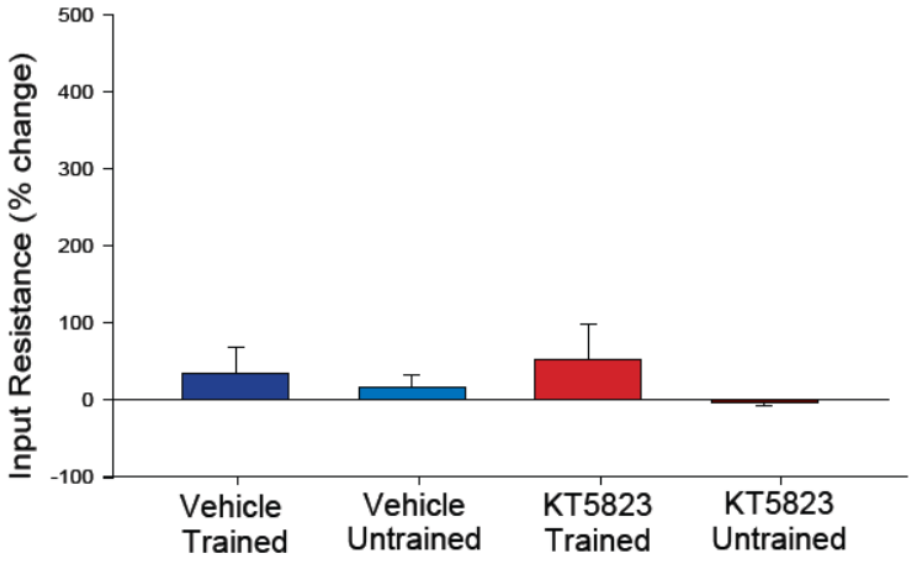


Figure 37 Role of KT5823 in B51 input resistance

TSN Excitability

There was a significant difference (p= 0.007) in TSN excitability (calculated as a percentage change) among the four groups 15-min post training (n=12 in all groups). Post-hoc analyses determined a difference in groups: vehicle/trained versus KT5823/untrained (q=4.631; p<0.05), vehicle/trained versus vehicle/untrained (q=4.258; p<0.05), KT5823/trained versus KT5823/untrained (q=4.838; p<0.05) and KT5823/untrained versus vehicle/trained (4.274; p<0.05) (Figure 38).

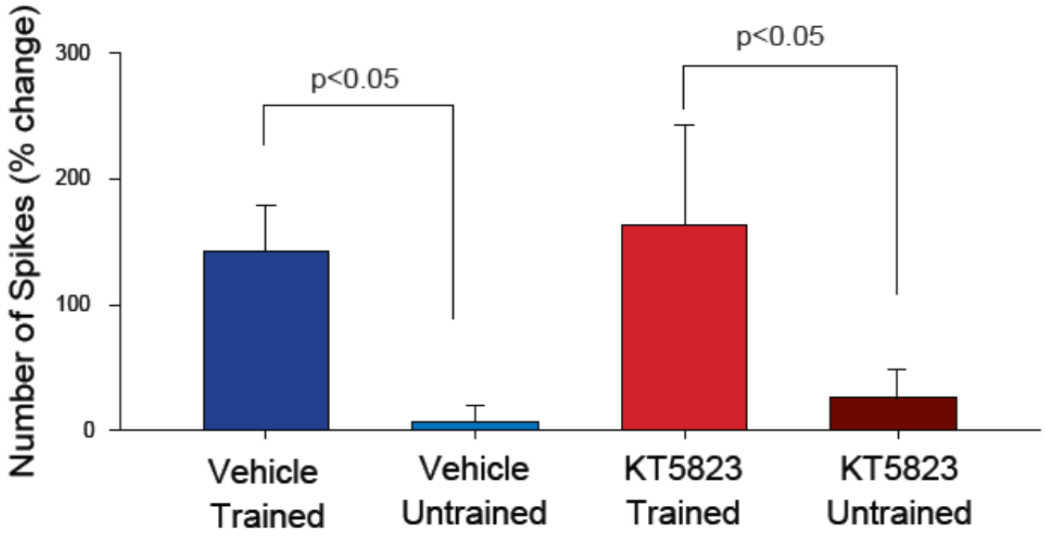


Figure 38 TSN excitability (measured as number of spikes % change)

Below are traces of TSN excitability illustrative of their respective group (Figures 39-42).

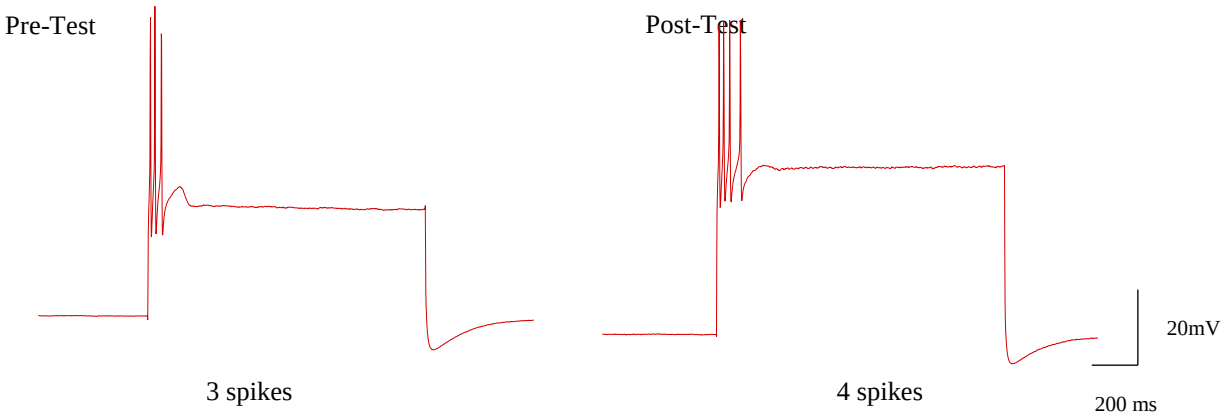


Figure 39 Intracellular recording representative of Vehicle Untrained (Pre-Test and Post-Test respectively) Preparations

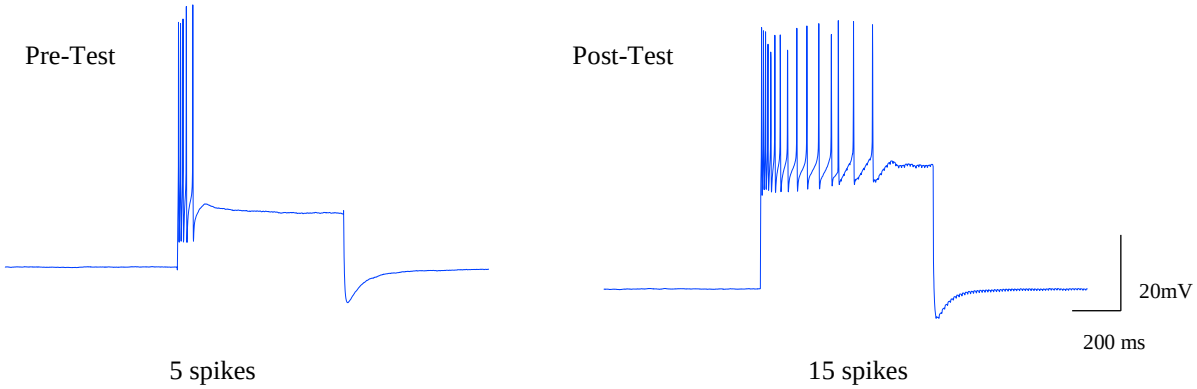


Figure 40 Intracellular recording representative of Vehicle Trained (Pre-Test and Post-Test respectively) Preparations

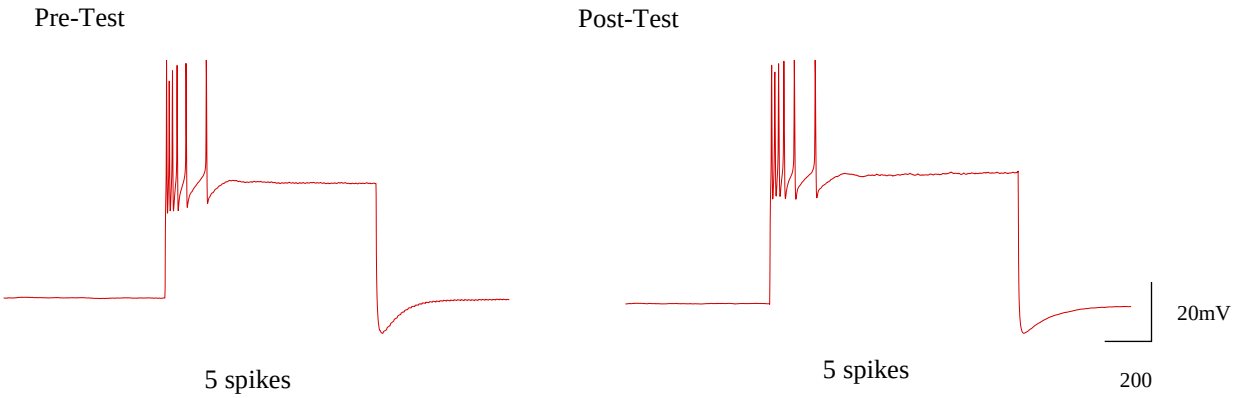


Figure 41 Intracellular recording representative of KT5823 Untrained (Pre-Test and Post-Test respectively) Preparations

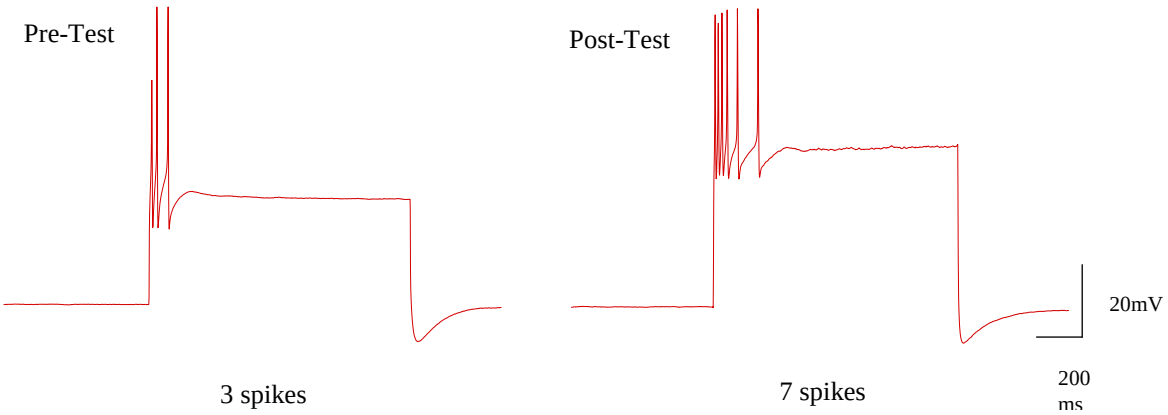


Figure 42 Intracellular recording representative of KT5823 Trained (Pre-Test and Post-Test respectively) Preparations

The resting membrane potential of the TSNs cells were also determined (see Figure 43). There was no overall significance among the four groups (H=1.656 with 3 degrees of freedom; p=0.647)

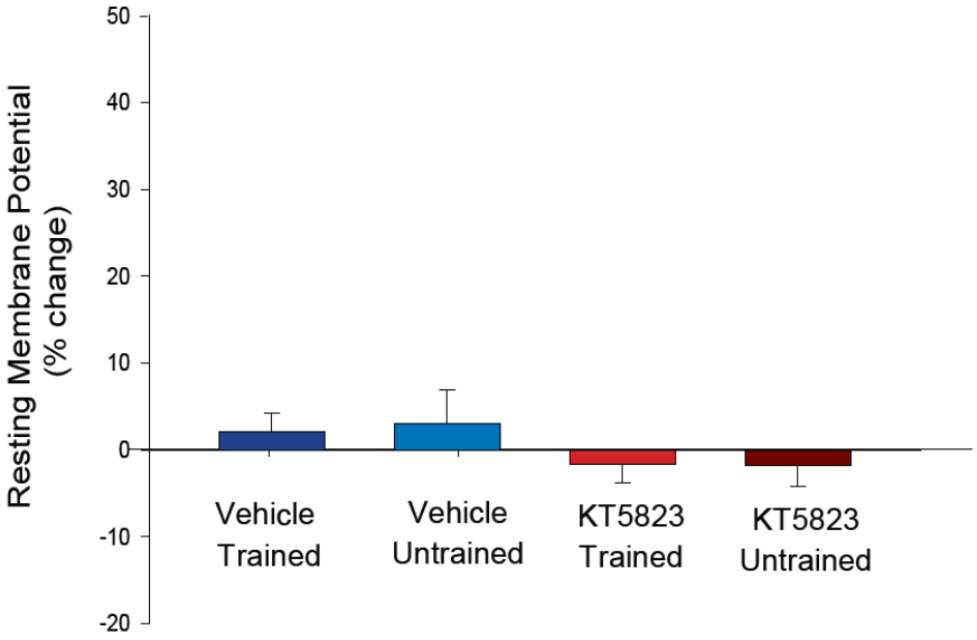


Figure 43 TSN resting membrane potential (measured as % change)

DISCUSSION

I tested the role of PKG in short-term and intermediate-term memory in *Aplysia* and found learning was blocked not only *in vivo*, but also *in vitro*. It is therefore unlikely that PKG plays a role in short-term and intermediate-term memory associated with the behavioral and neuronal plasticity when *Aplysia* is exposed to an aversive stimulus.

KT5823 did not block the training-induced feeding suppression

When *Aplysia* is exposed to an aversive stimulus, the animal decreases its non-defensive behaviors such as feeding, as the need to implement such a behavior during times of stress is not needed (Acheampong et al., 2012). As seen from several studies in *Aplysia* (Cleary et al., 1988; Acheampong et al., 2012; Shields-Johnson et al., 2013) including this one, feeding is one such behavior that constitutes a lesser precedence in the presence of an aversive situation. When the animals were exposed to aversive stimuli, their feeding decreased and subsided 15-min and 2-hr post the aversive stimuli exposure.

Protein kinase G was investigated as a potential molecule that may be responsible for these changes. The cGMP pathway dependent on PKG had been previously implicated in the behavioral and cellular plasticity that occurs as a result of exposure to aversive stimulus. Nitric oxide (NO) signaling is required for behavioral plasticity in *Aplysia* (Farruggella et al., 2015); specifically, NO signaling is necessary for long-term sensitization of the training-induced TSWR and is also required for the training-induced feeding suppression (Farruggella et al., 2015). As the likely downstream target along this pathway was PKG, this enzyme was therefore chosen to evaluate its role in these behavioral and accompanying neuronal changes that occur when the animal is exposed to an aversive stimulus.

The alternative hypothesis for this objective therefore indicated that KT5823 (blocking PKG) would prevent the training-induced feeding suppression. A number of studies demonstrated that the cGMP pathway dependent on PKG is necessary for the varying behavioral and neuronal plasticity associated with learning and memory. For example, the role of the NO/sGC (soluble guanine cyclase)/PKG on morphine-induced reward memory associated with a conditioned placed paradigm was assessed and found that the consolidation of this memory required the NO/sGC/PKG pathway associated with the morphine-related memory (Shen et al., 2012). Using mice as a model system, it was also identified that NO/cGMP/PKG pathway played a critical role in cognitive deficits and depression (Zhou et al., 2017). Additionally, Michel et al. (2011) demonstrated that PKG played a role in negatively reinforced operant memory in *Aplysia*.

Animals injected with the control solution (vehicle) exhibited the characteristic response: vehicle animals that were exposed to the training depicted a decrease in their feeding behavior 15-min and 2-hr post training exposure. Thus, the animals learned from the training to which they were subjected. KT5823 injected animals that were untrained did not exhibit a significant change in number of bites at either the 15-min or 2-hr posttest time points. This would be the expected result since they were not exposed to the training and so would not demonstrate a form of learning. However, the KT5823/trained animals responded in the similar way to the vehicle/trained animals: at the 15-min and 2-hr time post training these animals decreased their feeding behavior, indicating that the KT5823 did not block the learning.

These results suggest that PKG may not contribute to this training-induced feeding suppression. However, prior to ruling out that PKG was not the enzyme

responsible for these changes, it was important to assess if it had any influence on defensive behaviors, and, if directly bath-applying the drug to the nervous system would warrant any different results. This overall approach of studying different angles behaviorally and neuronally, would thus solidify the conclusion of whether PKG was responsible for these behaviors. One explanation of the negative result was that the concentration of 6.5 μM could have been lower than required to block PKG. Therefore, it was decided to test the effects of a doubled concentration of KT5823 (13μM) on feeding suppression induced by sensitization training.

Two-fold increase of KT5823 did not block the training-induced feeding suppression

Both vehicle/trained and KT5823/trained animals exhibited the training-induced feeding suppression and solidified the conclusion that KT5823 did not block the learning that manifested post training. It was then discussed that perhaps PKG may be involved in long-term memory instead of short-term memory. Instead of PKG, perhaps another molecule may be involved in the short-term memory accompanying these behavioral changes. It could also further probe the question of whether activation of other mechanisms would account for the memory that took place. However, before exploring other avenues, it was imperative to move forward with the different objectives of the project as were outlined to establish a well-rounded, solidified result.

KT5823 did not block the training-induced expression of sensitization

The role of PKG in the expression of sensitization was also examined. The learning that was identified in the vehicle trained animals was also demonstrated in the KT5823 trained animals. Using the original concentration of KT5823 (6.5 μM/100grams), it was investigated whether the KT5823 would block the sensitization

learning that the animal experienced when exposed to the training. Results from this second objective indicated that KT5823 did not block the training-induced expression of sensitization, implicating that PKG was perhaps not the molecule responsible for this behavioral change.

As a result of the negative data, it was speculated whether cGMP-PKG pathway is actually involved in long-term memory consolidation instead of the short-term memory that was investigated in this project. Studies from Michel et al. (2011) identified the involvement of the NO-PKG pathway for long-term memory formation involved in operant memory; perhaps PKG may follow a similar trend for non-associative learning. Also, Byrne and Hawkins (2015) emphasized several points when comparing short-term and long-term memory consolidation such as the fact that long-term memory requires gene translation and transcription. They accentuated the sustenance of not only intracellular but extracellular feedback loops which can account for the alteration of targeted molecules (Byrne and Hawkins, 2015). Perhaps, PKG is required to undergo prolong activation associated with these behavioral changes, longer than the short-term and intermediate-term points that were investigated in this study. Investigation on long-term non-associative memory on the behavioral changes described in this study are currently being conducted in the Mozzachiodi lab. Using KT5823 (6.5µM), Salas and Wainwright (2017) indicated that KT5823 blocked both long term feeding suppression (Figure 44) and sensitization (Figure 45), suggesting that the enzyme is required for the long-term memory associated with these behavioral changes (Salas et al., 2017, personal communication). Culmination of this study will help determine the role of PKG in behavioral and neuronal plasticity at the long-term time point. Current results suggest that

findings from this thesis and Salas et al. (2017, in progress) replicate results obtained from Michel et al. (2011), emphasizing that PKG may only play a role in long-term memory and not in short-term or intermediate-term memory.

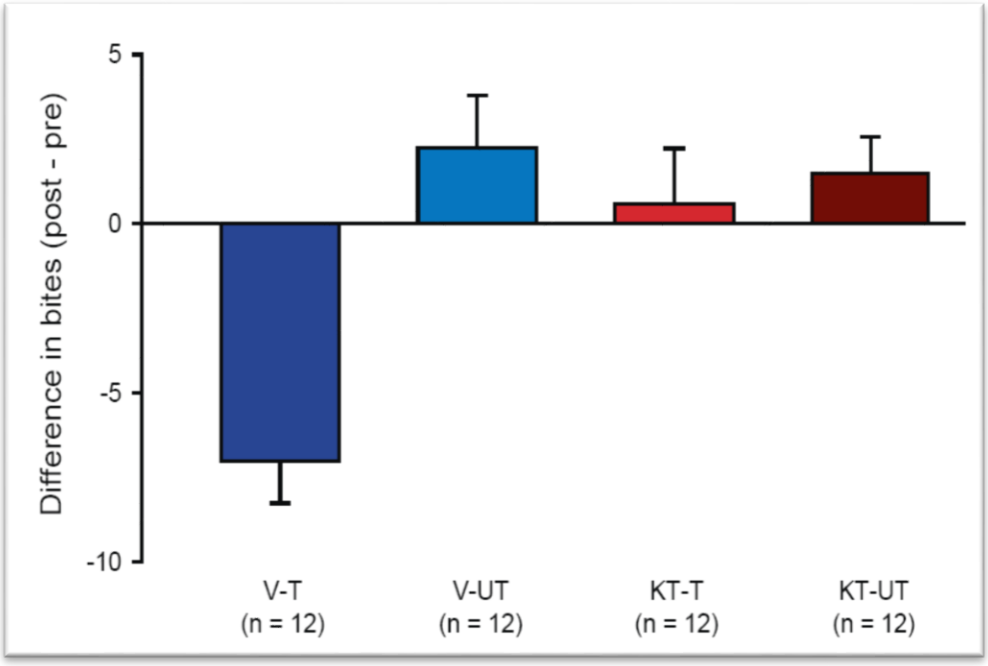


Figure 44 Preliminary data from Salas and Wainwright (2017) indicating feeding suppression blocked in KT-T (KT trained group) at 24-hr (Note Fig 44 & 45: V-T = vehicle/trained; V-UT=vehicle/untrained; KT-UT= KT/untrained)

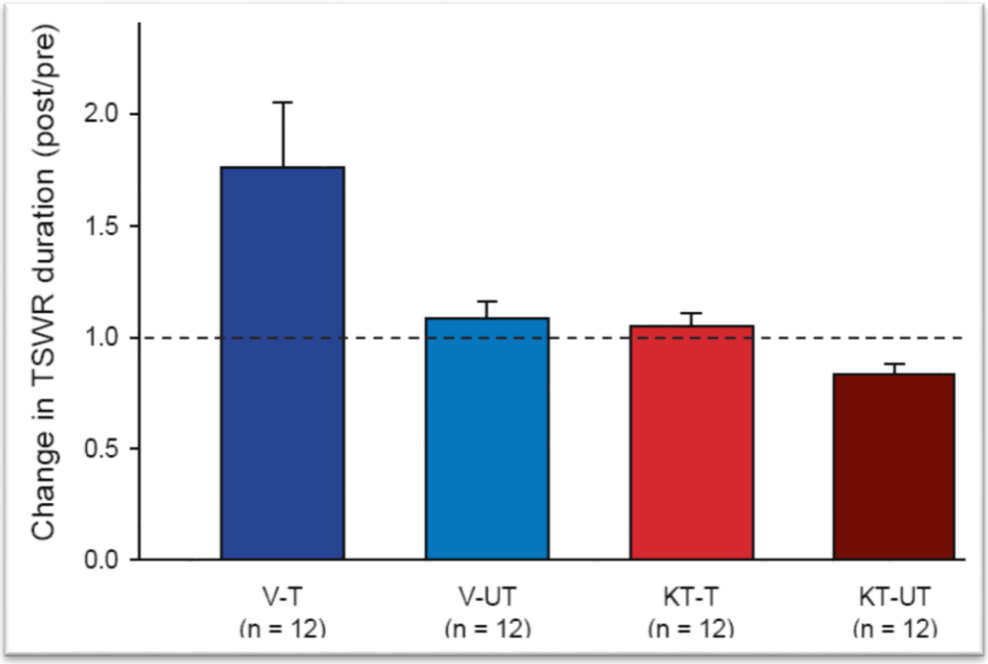


Figure 45 Preliminary data from Salas and Wainwright (2017) indicating sensitization blocked in KT-T at 24-hr

PKG may play a partial role in modulating the feeding circuity but not TSWR circuitry

While behavioral results were indicative of the fact that PKG may not be involved in the behavioral changes that occur when the animal is exposed to aversive stimuli, it was important to assess if similar results would be seen when directly subjecting the ganglionic nervous system to the drug. Therefore, to strengthen this this negative result, it was critical to conduct the study *in vitro* as *in vivo* results may be affected by a number of reasons such as the drug could have dissipated to different body parts of the animal or the animal itself could add the complication of modifying its own behavior in response to the drug. By directly bath-applying the drug, it was possible to allow the entire ganglionic system to be immersed in the vehicle or KT5823 solution. The two parts of the ganglionic nervous system that were assessed were the buccal ganglia containing neurons B51 and the pleural-pedal ganglia containing TSNs.

Decision-making neuron B51 is a characteristic component of the central pattern generator associated with feeding (Nargeot et al., 1999), which made it the ideal cell to study as it modulates the feeding behavior. This study focused on comparing and contrasting the excitability, measured as burst threshold, between and among vehicle trained/untrained and KT5823 trained/untrained preparations.

There was an increase in overall B51 excitability for the trained preparations. Although seemingly counterintuitive, the increase in excitability indicates that more current was required to induce a response from neuron B51. The behavioral correlate would indicate that there would be a decrease in the number of bites; the animal would require a stronger feeding incentive to initiate a bite. Similarly, neuron B51 required a higher current intensity to generate its plateau potential.

Both vehicle/trained and KT5823/trained preparations showed significant learning when compared to their respective control counterparts. While vehicle/trained preparations portrayed a training-induced decrease in B51 excitability, KT5823-preparations induced a lesser decrease in B51 excitability, indicating that KT5823 may have had a partial block on B51 decreased excitability. Perhaps, there could be the activation of PKG via more than one mechanism constituting other molecules such as cAMP (Burnette and White, 2006) or a combination of other protein kinases such as PKC/PKA (Hou et al., 2003).

The two other membrane properties that were measured in B51 were resting membrane potential and input resistance. As expected, input resistance remained unaffected among all four treatments, indicating that the sensitization training did not affect this membranal property. Interestingly, the statistical analysis determined by the SNK assessed that there was a difference among the treatments for the resting membrane potential: specifically of the KT5823 groups. This result suggested that perhaps there was an effect of the drug on this property as conversely, vehicle preparations did not experience this difference. Overall, analysis from B51 data suggest that while PKG may not be the singular molecule involved in modulating feeding, it does indicate that PKG may have a segmental role associated with this learning plasticity.

On the contrary, results from TSN excitability data indicated that KT5823/trained preparations did not have a block in the training-induced increased excitability. Based on the strong TSWR results, it was determined that the excitability was not blocked. The results obtained from this cellular correlate were analogous with the behavioral results in

that KT5823 did not block the training-induced increase in TSN excitability and training-induced expression of sensitization, respectively.

Conclusions and Future Directions

The overall result of this thesis indicated that PKG may not be the sole enzyme responsible for the behavioral and neuronal plasticity associated with aversive stimulus in short-term and intermediate-term changes in *Aplysia*. However, these results strongly constitute a framework by which future projects can be taken into consideration to narrow down other molecules and/or mechanisms responsible for these changes.

A primary approach that can be taken is to study PKG on long-term memory potentiation. Researchers Michel et al. (2011) identified that another pathway involved in memory formation- mitogen-activated protein kinase (MAPK) determined that PKG activity was required for MAPK activation requiring protein synthesis and translation but was not required for necessary for the early MAPK phase. The early MAPK phase, constituting the short-term memory, therefore was not dependent on the activation of PKG (Michel et al., 2011). Another study determined that the NO-cGMP-PKG pathway was involved for fear memory formation in the lateral amygdala of rats for long-term memory potentiation but not short-term memory (Ota et al., 2008).

Based on information gathered from studies such as those mentioned above, it can be hypothesized that the cGMP-PKG pathway may be involved in long-term memory in the behavioral and neuronal plasticity that were discussed in this thesis. Therefore, the next plausible step for this study would be to conduct the behavioral and corresponding neuronal investigations examining long-term memory.

Additionally, other avenues can also be taken to assess various mechanisms in controlling these plastic modifications. Since the neuronal result that was obtained from this thesis indicated that KT5823 constituted a partial block in the decrease in B51 excitability, it can be suggested that other mechanisms may be involved in conjunction with the cGMP-PKG pathway. For example, researchers Burnette and White (2006) suggested that PKG can undergo cross activation with CAMP. Other researchers determined that PKG can be directly activated through another enzyme PKC (Hou et al., 2003). Furthermore, the enhancement of the long-term memory consolidation via the cGMP-PKG pathway also requires the signaling of cAMP-PKA signaling (Bollen et al., 2014). Therefore, perhaps, there is a combination of mechanisms that can constitute for the learning and memory in *Aplysia* as well.

Moreover, other downstream targets of the cGMP pathway could be investigated: for example, cyclic nucleotide-gated ion channels (Lopez-Jimenez et al., 2012). Another option could be to investigate the regulation of several phosphodiesterase molecules such as PDE9 or PDE2 that have been identified to be involved in long-term memory potentiation (Reierson et al., 2011). These examples constitute the fact that there could be other downstream actions that could affect the neuronal plasticity. Additionally, it also suggests that PKG can affect the behavioral and neural plasticity through not one mechanism alone but perhaps through an interaction of different mechanisms.

This study aimed to identify the role of a particular enzyme, PKG, and its involvement on behavioral and neuronal plasticity when *Aplysia* is exposed to aversive stimuli. Although PKG did not have a direct role in the behavioral plasticity, the neuronal investigation indicated that PKG may have a partial role in the training-induced change in

B51 excitability. Further analyses will be required to determine the involvement of PKG in training-induced B51 decreased excitability for short-term and intermediate-term memory. While future work may consist of analysis of other target molecules, current directions include assessment of PKG in long-term memory associated with training-induced feeding suppression and sensitization.

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