INFRARED NEURAL STIMULATION OF *APLYSIA CALIFORNICA*

By

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Introduction

Nervous System Overview

The nervous system is a complex, highly specialized system that is organized into the central and peripheral nervous system. The central nervous system (CNS) is comprised of the brain and the spinal cord, which are housed in the bones of the cranium and vertebral column, respectively. The peripheral nervous system (PNS) is made up of nerve fibers that carry information between the CNS and other parts of the body such as muscles, skin, and other organs. Within the PNS, the afferent division carries information either from the external environment or the internal peripheral environment to the CNS. The efferent division of the PNS carries information from the CNS to the effector organs, which can be muscles or glands. The efferent nervous system is split into the somatic nervous system and the autonomic nervous system. The somatic nervous system contains motor neurons that innervate skeletal muscles, while the autonomic nervous system contains neurons that innervate smooth muscle, cardiac muscle, and glands (Sherwood, 2007).

The tissue of the nervous system is made up of nerve cells and nerve fibers. Nerve cells are found in primarily in the CNS while nerve fibers are found in both the CNS and PNS. A ganglion is a group of nerve cells that exist outside of the brain or spinal cord and are surrounded by a fibrous sheath. The smallest functional unit of the nervous system is the neuron. A neuron is composed of dendrites and a cell body that receive signals from

other neurons, the axon hillock that initiates the electrical signal, the axon that conducts the signal, and the axon terminals that serves as the output zone to influence surrounding cells as shown in Figure 1.

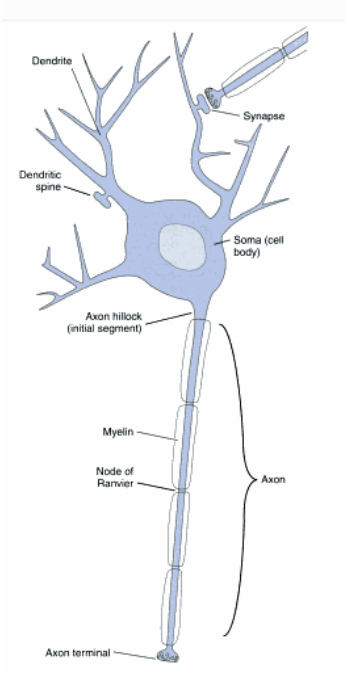

Figure 1: Structure of a myelinated neuron. The neuron is the smallest functional unit of the nervous system (RA Rhoades, RB Bell, 2009).

The neural tissue that comprises the nervous system is excitable tissue. All excitable cells possess a membrane potential due to separation of positive and negative charge across a semi-permeable membrane. Excitable tissues produce electrical signals, which are rapid, transient changes in membrane potential that propagate as a result of changes in ion concentration on either side of the plasma membrane. Nerve cells transmit information via these electrical signals. A local change in membrane potential causes a graded potential which can occur at varying degrees of magnitude depending on the type and size of the stimulus causing the initial membrane potential change. If the membrane

potential exceeds a critical level called the threshold potential, the potential will rapidly change and reverse resulting in an action potential. Depolarization, or the reduction in the magnitude of the negative membrane potential, occurs with an influx of sodium ions and an outflow of potassium ions from the cell due to the subsequent opening and closing of voltage gated ion channels. The permeability and conductance of the membrane to these ions increases in a positive feedback loop causing an explosive depolarization, initiating the action potential. This impulse automatically conducts through the neuron via continuous conduction or saltatory conduction. As shown in Figure 2, continuous conduction is the propagation of the action potential down the full length of membrane making up the neuron's axon due to the spread of current from active areas undergoing an action potential to surrounding inactive areas. Some nerve fibers in the PNS and CNS experience a faster method of propagation called saltatory conduction. Nerve fibers covered in myelin, which is a lipid produced by oligodendrocytes in the CNS and Schwann Cells in the PNS, have an increased speed of action potential conduction due to insulation of the membrane. Instead of spreading through the entire membrane as in continuous conduction, current can jump between unmyelinated regions at the Nodes of Ranvier where there is a concentration of voltage gated sodium channels (J Seifter, A Ratner, D Sloane, 2005).

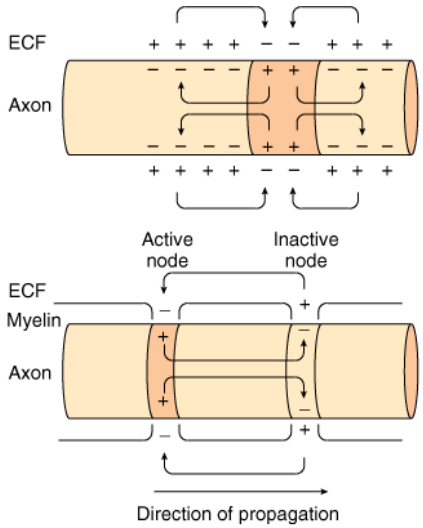

Figure 2: Current spread in an unmyelinated nerve fiber through continuous conduction (above) and a myelinated nerve fiber through saltatory conduction (below) (K.E. Barrett, S.M. Barman, S. Boitano, H. Brooks, 2009).

Action potential propagation occurs due to changes in membrane permeability in response to a triggering event. An intrinsic triggering event may be a change in the electrical field, chemical messenger on a surface receptor of the nerve, mechanical waves, or an imbalance in the ion pump. There are two types of membrane channels, leak channels and gated channels that are responsible for permitting ion passage in response to a triggering event. Leak channels are constantly open and allow unregulated ion passage through the membrane. Gated channels are transmembrane proteins that open or close due to a conformational change initiated by a triggering event. Voltage gated channels, chemically gated channels, mechanically gated channels and thermally gated channels are the primary channels capable of responding to the triggering event and allowing ion movement and subsequent membrane potential fluctuation.

Neural Stimulation Techniques

Neural stimulation can be defined as the process of initiating action potentials in peripheral and central nerves and neurons through an external energy source (J. Wells, et al, 2005b). A safe and effective method of neural stimulation is necessary in both a laboratory and clinical setting. In the research laboratory, neural stimulation can be used to study the connections in neural networks and gain a better understanding of the anatomy and physiology of the nervous system. Clinically, neural stimulation is relevant to the development of therapeutic and diagnostic tools to treat neural disorders. Among these neural disorders are sensorineural deafness, epilepsy, paralysis, chronic pain, and peripheral neuropathy.

Electrical Stimulation

Electrical stimulation has long been held as the gold standard for neural stimulation. One of the first reports of electrical stimulation occurred in the late 18th century when Louigi Galvani discovered that applying charge to the nerves of a detached frog leg induced a muscle twitch (L. Galvani, 1953). Since then, electrical stimulation has widespread application in the laboratory and for use in neural implants and other therapeutic and diagnostic tools (Fritsch and Hitzig 1870; Geddes and Bourland 1985; Devinsky 1993). To stimulate a nerve electrically, one or more electrodes are placed in contact with the nerve, and a small current is applied. The injected current causes a flow of ions, which results in the depolarization of the nerve membrane. If the level of injected current causes a depolarization that exceeds threshold, an action potential will propagate

down the nerve fiber. Electrical stimulation of the frog sciatic nerve is shown in the trace in Figure 3.

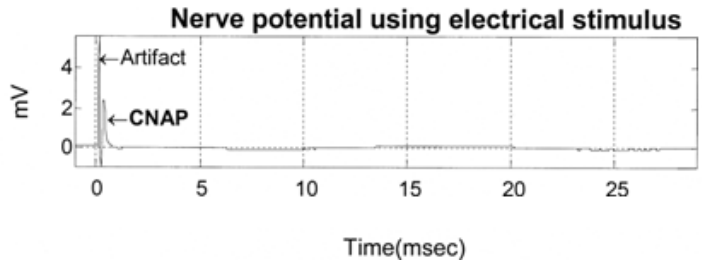

Figure 3: Electrical stimulation of the frog sciatic nerve yields a compound nerve action potential (CNAP). The recording is obscured by the presence of a stimulation artifact (J Wells et al., 2005a).

Electrical stimulation can be applied to the brain, peripheral nerve fibers, or sensory systems to evoke action potentials. It has also been applied to cardiac tissue to achieve cardiac pacing and ventricular defibrillation to eliminate abnormal heart rhythms (L.A. Geddes, 1985). The recent development of neural prostheses uses electrical activation of neural tissue to restore function to individuals with neural damage. Applications include restoration of limb movement following spinal cord injury, restoration of bladder function, and incorporation into cochlear implants (W.M. Grill, 2000). Electrical stimulation is the technology used in deep brain stimulators to treat tremors associated with movement disorders such as Parkinson's disease and dystonia. Cochlear implants have effectively utilized this modality to stimulate hair cells for the restoration of hearing loss. Electrical stimulation is also used to identify the functionality and connectivity in specific regions of the nervous system (H. Ueno et al., 2001)

Electrical stimulation is a very well characterized modality. It has the advantages of being reliable and having easily controllable parameters such as current, voltage, repetition rate, and pulse duration. Compared to other stimulation modalities, electrical

stimulation is spatially precise. There have been significant advances in the design of electrodes and the delivery of the electrical stimulus to provide precise and accurate stimulation (J. Wells et al., 2007). Although the characteristics of electrical stimulation have made it widely used in the laboratory and clinic, there are distinct shortcomings. Stimulating electrically necessitates direct contact with the tissue. Extracellular stimulation requires a hook electrode to be wrapped around the tissue, and intracellular recording requires the impalement of a cell. The physical contact with the tissue as well as the toxicity of the electrode material may cause irreversible damage to the tissue (D.R. Merrill et al., 2005). Spatial resolution during electrical stimulation is dependent on electrode configuration. In the simplest configurations, changes in transmembrane potential are greatest closest to the stimulation electrode and decreases in amplitude as distance increases. Current spread can cause difficulties when attempting to activate a localized group of fascicles or neurons. More complex electrode configurations are required to stimulate small regions of the tissue. Furthermore, electrical stimulation causes a stimulation artifact because stimulation and recording of the signal are done in the same domain. The presence of a stimulation artifact is a disadvantage in the laboratory because it masks the signal, making it difficult to analyze (J. Wells et al., 2007c).

Other Stimulation Modalities

In addition to electrical stimulation, magnetic, chemical, thermal, and mechanical methods have been utilized for the stimulation of neural tissue. Similarly to electrical stimulation, each of these modalities has its advantages and shortcomings. Magnetic stimulation of the frog peripheral nervous system was first seen in the late 1950's.

Sinusoidal magnetic fields can be utilized to produce a visible muscle contraction. In the brain, transcranial magnetic stimulation is a non-invasive, safe, and painless technique for activating regions in the brain. This method has the ability to modify cortical activity, which is a benefit for the investigation of cognitive function and the development of therapeutic tools (P. Anninos et al., 2006). However, the magnetic field generated during magnetic stimulation is not focal and yields a spatial resolution of a few millimeters. Regardless of the configuration of source coils, the spatial distribution of the field strength cannot be effectively concentrated. This resolution is not high enough to stimulate individual fascicles or nerve cells (V. Walsh and A. Cowey, 2000). Chemical stimulation can be applied to neural tissue by inducing chemical reactions that allow for the repeated induction of action potentials. It has been shown that changing the concentration of ions normally present in the extracellular fluids through the administration of a chemical stimulus can induce action potentials. This method of stimulation is highly non-specific due to the diffusion of solution outside a region of interest (R. Orchardson, 1978). Mechanical stimulation is another method to activate neural tissue that provides a greater localization of excitation than chemical or magnetic means of stimulation. Previous studies have shown that the propagation of ultrasonic waves in the presence of a strong magnetic field can induce action potentials in cortical tissue (S.J. Norton, 2003). Thermal means of stimulation have been shown through the use of continuous wave lasers to increase the nerve temperature above a threshold to cause stimulation. However, thermal buildup may cause damage to the tissue (S. Tozburun et al., 2010).

Optical stimulation can also be achieved using various wavelengths of ultraviolet, visible, and infrared light. Single neuron stimulation using a continuous wave He-Ne laser with wavelength in the visible spectrum was reported (P. Balaban et al., 1992). A short UV, pulsed excimer laser was reported to stimulate nerve fibers (G. Allegre et al., 1994). A mode-locked high intensity infrared femtosecond laser was used to stimulate neurons in the somatosensory cortex (H. Hirase et al., 2002). Optogenetics is another method of optical stimulation where visible light is used to stimulate channel-rhodopsin channels that have been genetically engineered into cells (E.S. Boyden et al., 2005). However, this technique does not rely on the intrinsic sensitivity of the tissue. Most recently, a technique was established using pulsed infrared light to stimulate neural tissue.

Infrared Neural Stimulation

In recent years, a method of optical stimulation called infrared neural stimulation (INS) has been proposed as an alternative method to electrical stimulation. Action potentials can be induced in the central and peripheral nervous system using low energy, pulsed infrared light (J. Wells et al., 2005a). While the benefits of electrical stimulation are not to be diminished, INS can provide another avenue to study and treat neural tissue because it does not include many of the impediments inherent to electrical techniques. Stimulation using infrared laser light does not require direct contact with tissue (J. Wells et al., 2005b). Laser light does not produce a stimulation artifact in the recording domain, making nerve signals easier to study. Spatially selective stimulation is an inherent characteristic of this technique and does not require complex electrode configurations. A direct comparison of electrical and optical stimulation modalities illustrates these distinct

advantages in Figure 4. This figure shows that electrical and optical stimulation yield signals of a similar shape and timing. However, INS can target individual fascicles, whereas the depicted electrode configuration does not allow this. Additionally, at threshold, the magnitude of the signal is larger for electrical stimulation than INS due to a smaller number of axons being recruited during infrared stimulation.

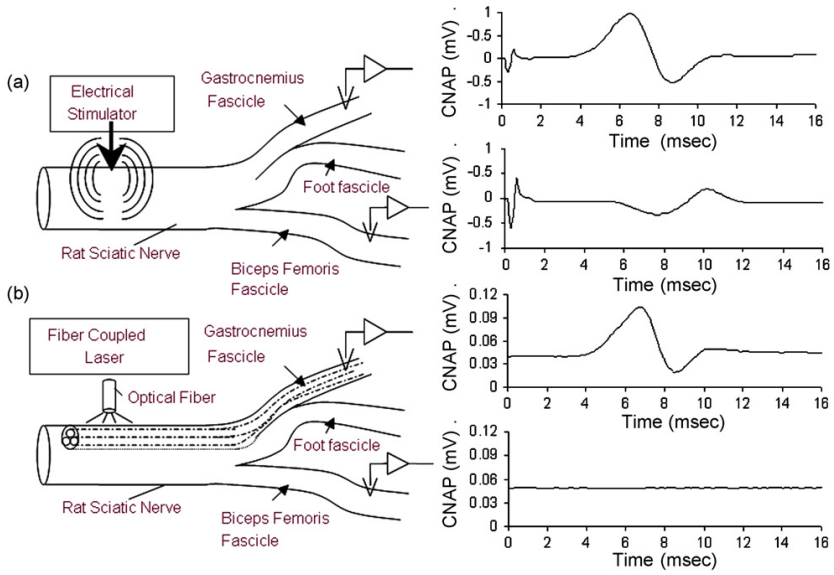

Figure 4: Neural stimulation at threshold using a) electrical stimulation yields a stimulation artifact as well as the activation of undesired fascicles. b) INS shows artifact-free, spatially selective stimulation. CNAP = Compound Nerve Action Potential (J. Wells et al., 2007a).

Previous studies have demonstrated that the optimal stimulation wavelengths in the infrared region of the electromagnetic spectrum are highly dependent on the absorption spectrum of water. Neural tissue is primarily water, which absorbs emitted light energy and converts it to heat energy. The overarching mechanism of INS is the establishment of a thermal gradient in the cell membrane to initiate action potentials. The underlying physiological mechanism remains unknown (J. Wells et al., 2007c). Wavelengths of 1.85 and 2.1 μm correspond with relative peaks on the water absorption

spectrum, which yield the largest window for stimulation. The safety of stimulation at these wavelengths is quantified by taking the ratio of ablation radiant exposure levels to threshold radiant exposure levels (J. Wells et al., 2005a; 2005b).

Previous work and applications of INS

Previous work was done on a variety of model systems to characterize and apply the INS. Studies demonstrate the ability to effectively stimulate spatially specific regions of neural tissue with pulsed infrared light in amphibian and mammalian peripheral nerves without inducing damage (J. Wells et al., 2005a; 2005b). Recent work has shown effective stimulation in the auditory nerves and the spiral ganglia of the rat cochlea for restoration of hearing as well as the cavernous and facial nerve for its preservation during surgical resection of a cancerous prostate gland (A.D. Izzo et al., 2006; N.M Fried et al., 2008; I.U. Teudt et al., 2007). More recent work has concentrated on applications of INS in the central nervous system. Studies show that INS has an overall inhibitory effect in the neural activity of the somatosensory cortex (J.M. Cayce et al, 2010; 2008). In the heart, INS was demonstrated to pulse lock the beating of quail hearts *in vivo* (M. Jenkins et al., 2010).

With the knowledge that INS works in a variety of applications, studies have also been done on the mechanism by looking at the photobiological effects of light absorption. Photochemical, photomechanical and photothermal mechanisms were investigated as possible photobiological effects. These studies showed that a thermal gradient in the target nerve is responsible for nerve excitation through INS. Infrared light deposited into the tissue from the laser is absorbed and converted to heat to cause action potential

propagation. It is hypothesized that the physiological mechanism by which this is accomplished is either through increasing channel conductance or the activation of heat sensitive channels. However, the exact physiological mechanism is still unknown (J. Wells et al., 2007c).

Motivation

Before INS can be used clinically in human patients, there must be a clear understanding of its underlying physiological mechanism. Understanding the underlying mechanism is an integral step towards INS becoming a clinical alternative to electrical stimulation. If researchers and clinicians know how it works, its safety and utility can be more accurately determined. Although studies have shown that neural activation with pulsed infrared light occurs by a transient thermally mediated mechanism, it is still not known how the thermal gradient is affecting the neural tissue on a molecular level. While implementation of INS has been done in a variety of animal models including rats, frogs, cats, primates, and some human subjects, the question of mechanism is most easily answered in a lower order organism. Studies done on the invertebrate marine mollusk, *Aplysia californica*, will allow a step back to answer more basic questions about INS. Studies on this lower order organism will allow the further refinement of optimal laser parameters. The optimal laser parameters must be known before this modality can be implemented successfully in patients without inducing damage to the tissue. Researchers will benefit from understanding the mechanism because it will open up new applications for the use of infrared light in neural tissue. This modality can be used for researchers and clinicians without the presence of a stimulation artifact that clouds the desired electrophysiological signals.

Aplysia californica

The marine mollusk, *Aplysia californica*, is commonly used as a model organism in neurobiology. An invertebrate animal model is a good choice for studying the underlying physiological mechanism and the optimal laser parameters for INS. While mammalian animal models may more closely resemble humans, one advantage of performing studies in *Aplysia* is that it has a relatively experimentally tractable, less complex nervous system. The human nervous system has on the order of 100 billion neurons within its central nervous system, but *Aplysia* have only about 20,000 neurons which are organized into nine ganglia (K.E. Cullen, 2009). The specific ganglion used in a majority of the studies described here is the buccal ganglion, which controls the animal's feeding apparatus called the buccal mass. The buccal ganglion is well characterized in neurobiological literature, which includes extensive mappings of the location of neurons and the numerous synaptic connections between the individual nerves (S.C. Rosen, 1991). This allows repeatable stimulation and identification of action potentials from individual cells. *Aplysia* are a relatively robust system both *in vivo* and *ex vivo*. *Ex vivo* studies can be performed for an entire day with healthy nerve functioning. Other advantages of the *Aplysia* model are that it has a short life cycle and is relatively easy and inexpensive to raise. Fewer ethical concerns are raised in studying invertebrates allowing for experimentation to proceed at a faster rate. Further studies concentrated on more complex behaviors such as feeding are easily studied in the *Aplysia* system because *Aplysia* are easily conditioned in response to a stimulus.

Mammalian nerves are myelinated, which allows faster action potential propagation due to saltatory conduction. *Aplysia* nerves are unmyelinated, meaning that

the method of action potential propagation occurs by a much slower process. The differences and similarities of this invertebrate must be taken into account during experimentation and analysis. By studying the response of *Aplysia* to INS, we can make predictions about how neural tissue in humans will operate in response to infrared light.

Hypothesis and Objectives

The applications of infrared nerve stimulation are rapidly expanding, from use in the rat sciatic nerve (J. Wells et al., 2005a), cochlea (A.D. Izzo et al., 2006), central nervous system (J.M. Cayce et al., 2010) and recent uses in the quail heart (M.W. Jenkins et al., 2010). Before INS can be safely implemented in human patients for these applications, researchers must have a thorough understanding of its underlying physiological mechanism. Previous studies done in the Vanderbilt Biomedical Optics Laboratory have shown that the mechanism of INS in the rat sciatic nerve is through the deposition of a thermal heat gradient (J. Wells et al., 2007c). However, it is not known exactly how the heat gradient causes action potential initiation. Additionally, the optimal laser parameters for stimulation must be solidified before clinical use. The hypothesis of these studies is that INS can be used to stimulate the neural tissue of *Aplysia californica* for the eventual study of INS mechanism and optimal laser parameters. The studies done here will lay the groundwork for future work in *Aplysia* by characterizing the normal functioning of the animal model. Due to the simpler, more experimentally tractable nervous system of invertebrates, we predict that *Aplysia* will be a good means for further exploration of questions concerning mechanism and optimal laser parameters.

This study has three objectives. First, the feasibility of stimulating neural tissue using INS in the buccal ganglion of *Aplysia californica* will be shown. Once the feasibility of INS has been established in this model, characterization studies will be performed to determine how INS pulse duration, repetition rate, wavelength, and ambient temperature affect stimulation thresholds. Finally, some preliminary studies will show that INS can be used to induce complex behavioral patterns. This thesis focuses on establishing the effects of INS in *Aplysia* through feasibility, parameteric, and behavioral studies using extracellular recording methods. The studies performed here will provide a solid foundation for further work done on a cellular level to determine how heat from INS activates neural tissue.

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CHAPTER II

Infrared Neural Stimulation of *Aplysia californica ex vivo*

Introduction

Recently, infrared neural stimulation (INS) has been shown to activate action potentials in the central and peripheral nervous system using an energy source in the infrared region of the electromagnetic spectrum. This technique can be used as an alternative to electrical stimulation, which is the gold standard for neural stimulation. The use of lasers to stimulate neural activity has several advantages over electrical stimulation: it is spatially and temporally precise to allow the stimulation of single nerve cells or fibers, it does not produce a stimulation artifact that obscures data acquisition, it requires minimal contact with neural tissue, and it has easily controllable parameters such as pulse duration and repetition rate. Stimulation of neural tissue is important both clinically and in the laboratory. In the lab, neural stimulation is necessary to study the functional connections of neural networks. A better understanding of the nervous system allows for the translation of this technology to patients who suffer from neural disorders such as epilepsy, sensorineural deafness, paralysis, chronic pain and many others. The ability to regain control over malfunctioning neural circuitry would provide treatment of neural disorders and greatly alter the quality of life for many people.

Previous studies of INS have shown that it is possible to induce a muscle twitch by stimulating the rat sciatic nerve *in vivo*, stimulate action potentials in thalamocortical brain slices of the central nervous system, and induce auditory signals by stimulating spiral ganglion cells in the cochlea (J. Wells et al., 2005b; J.M. Cayce et al., 2010; A.D.

Izzo et al., 2007). Although these studies have shown that stimulation is effective at optimal wavelengths in the infrared (2.1 μm and 1.875 μm), the physiological mechanism of this process is not fully understood. The pulsed infrared light is understood to cause stimulation by a transient thermally mediated mechanism, but the cellular and molecular processes by which this occurs are not yet known (J. Wells et al., 2007c). Awareness of the underlying processes involved is a necessary step before INS achieves full use in clinical applications because it will allow us to understand the full range of use and limitations of this modality.

In order to study the physiological mechanism of INS, we have chosen the marine mollusk, *Aplysia californica*, as the model organism due to its well-characterized and experimentally tractable nervous system. The purpose of this study was to show feasibility and characterize how INS works in the *Aplysia* so that INS as a whole can be better understood.

Methods

Studies were done *ex vivo* on the buccal ganglion in *Aplysia californica* ranging from 150 to 400 grams. Depending on the time of year, the animals were acquired from the wild or from a laboratory that raises them from eggs (Marinus Scientific, Long Beach, CA; National Resource for Aplysia, Miami, FL). Animals raised in the wild tended to be larger, more robust animals with wider diameter nerves than those raised in a tank. Animals were anesthetized through the injection of 333 mM isotonic magnesium chloride solution (0.5 mL/g) with the specific volume depending on the size and activity level of the animal prior to anesthetization. A sagittal cut was made from the rhinophores to the

top of the oral tentacles with the scissors angled upwards at 30 degrees to avoid damage to the internal tissues. The buccal mass, which is the muscle mass responsible for feeding, was removed with the buccal ganglion still attached. After the surgery, the buccal mass was bathed in *Aplysia* saline containing the following concentrations: 460 mM NaCl, 10 mM KCl, 22mM $MgCl_2$, 33 mM $MgSO_4$, 10 mM CaCl2, 10 mM glucose, and 10 mM MOPS buffer, set to a pH of 7.5. The buccal mass was placed under a dissecting microscope in a Sylgard lined Petri dish. The buccal ganglion was removed and pinned to the dish using 0.10 mm diameter Austerlitz stainless steel minutien insect pins (Fine Science Tools, Foster City, CA).

Feasibility Studies

Initial feasibility studies were performed at Case Western Reserve University. In these studies, the thin collagenous sheath overlaying the nerve cells was thinned and in some cases removed to allow for the acquisition of intracellular recordings. Extracellular recordings of action potentials were made by positioning an extacellular glass electrode over a neuron that projected from Buccal Nerve 2 (BN2) or Buccal Nerve 3 (BN3). All extracellular electrodes were made from single-barrelled capilary glass (A-M Systems, Everett, WA), pulled on a Flaming–Brown micropipette puller (model P-80/PC; Sutter Instruments, Novato, CA). Electrodes had a tip diameter of 40 μm and a resistance of 4.5 MΩ. In experiments where the sheath was removed, an intracellular electrode impaled the cell to measure intracellular membrane potentials. Intracellular recording electrodes were also made from single-barrelled capillary glass (AM Systems, Everett, WA) pulled on a Flaming–Brown micropipette puller (model P-80/PC; Sutter Instruments, Novato,

CA). Their resistances were 3–6 MΩ. The bridge was balanced for both stimulation and recording. Intracellular signals were amplified using a DC-coupled amplifier (model 1600; A-M Systems, Carlsborg, WA). Electrical recordings of action potentials were made at the cut end of BN3 on one hemi-ganglion using tightly fitting suction electrodes made from polyethylene tubing (Becton Dickinson, Sparks, MD). All polyethylene electrodes were made by pulling tubing over a flame and trimming the end such that the inner diameter of the electrode closely matches the diameter of the nerve fiber. A micromanipulator was used to position the electrode next to the cut nerve for suctioning. Nerve action potential responses to optical stimulation were recorded with the AxographX data acquisition software (AxographX, Sydney, Australia). Action potential recordings began before the laser was turned on and ended after twenty laser pulses were fired. All signals were filtered with a band pass filter of 300 to 500 Hz, and an amplification of 1000x was applied using an AC-coupled differential amplifier (model 1700; A-M Systems, Carlsborg, WA). The signal was digitized and analyzed using Axon Digidata 1322A data acquisition system (Molecular Devices, Sunnyvale, CA). For the initial feasibility studies, an infrared diode laser (Aculight Capella, Lockheed-Martin, Bothell, WA) was used for the optical stimulation of nerve cells. By varying the diode temperature, the laser can output wavelengths between 1.844 – 1.885 μm. All feasibility experiments were conducted at a wavelength of 1.875 μm. The threshold for stimulation was approximated in each experiment by starting at low peak power, pulse width, and repetition rate. Each parameter was systematically increased until nerve signals were induced.

Characterization studies

All characterization experiments in *Aplysia* were conducted at the Vanderbilt Biomedical Photonics Laboratory. Characterization experiments were conducted at a wavelength of 1.875 μm or 1.865 μm using an infrared diode laser (Lockheed-Martin Aculight, Bothel, WA) or 2.1 μm using a Holmium:YAG laser (Schwartz Electro Optics, Inc., Orlando, FL). Repetition rates were selected between 0.5 Hz and 10 Hz, and pulse durations were selected between 350 μs and 20 ms. The temperature of the bath was set to 0, 20, or 38 degrees Celsius. Using a fine micromanipulator, a 200 μm core flat polished optical fiber (Ocean Optics, Dunedin, FL) was positioned in contact with BN3 with the tip completely immersed in saline. Because the tip of the optical fiber was in direct contact with the nerve, the spot diameter was assumed to be 200 μm.

Repetition rate optimization was performed using repetition rates of 0.5, 1, 2, 3, and 10 Hz. INS at each repetition rate was performed using 3 ms pulse durations at a wavelength of 1.875 μm while the ganglion was immersed in a 20 degree Celsius saline bath. Each repetition rate was used to stimulate at least 3 healthy BN3 at 3 – 5 locations depending on the quality of the nerve (n = 12 – 18). Pulses of infrared light were delivered to the nerve an average of 1.5 mm away from the tip of the suction electrode. An initial approximation of the stimulation threshold for a given repetition rate was made by dialing up the energy level while recording in real-time. The energy level that first yielded an action potential in response to the succession of laser pulses was approximated as the stimulation threshold. To investigate how the threshold for stimulation changes with varying repetition rates, a repetition rate between 0.5 – 10 Hz was chosen at random and stimulated at a randomly chosen energy level slightly above or below the estimated

threshold point. The order of repetition rates and energy levels tested were randomized prior to experimentation to avoid conditioning of the nerve response. For the purposes of this study, stimulation was defined as 100% action potential firing consistency for twenty consecutive laser pulses. For each train of twenty laser pulses at a given repetition rate and energy level, a yes or no response was recorded with regards to whether or not each pulse induced an action potential. The energy of each laser pulse was measured immediately after the experiment using a pyroelectric detector and energy meter (PE50BB and Laserstar, Ophir, Jerusalem, Israel). All energy measurements were converted to radiant exposure (J/cm^2) using a spot size of 200 μm. Once this data was collected, a data analysis software package called PROBIT, developed at the Ultrashort Laser Bioeffects Program at Brooks Air Force Base in San Antonio, TX, was used to statistically determine a stimulation threshold for each repetition rate tested. The PROBIT software provided an output of the probability of stimulation versus radiant exposure in addition to the 10% and 90% fiducial limits of the analyzed data. The stimulation threshold is calculated to be the radiant exposure level that gives a 50% probability of ablation (ED_{50}).

Pulse duration optimization was studied by performing the same set of experiments with a wavelength of 1.875 μm, repetition rate of 1 Hz, at a temperature of 20 degrees Celsius for the following pulse durations: 2.5, 3, 4, 5, 6, 10, and 20 ms. A lower pulse duration of 350 μs was also tested using the Ho:YAG laser with a wavelength of 2.1 μm. Each pulse duration was used to stimulate at least 3 healthy BN3 at 3 – 6 locations depending on the quality of the nerve (n = 12 – 18). Wavelength optimization was studied by holding the repetition rate at 1 Hz, the pulse duration at 3 ms, the bath temperature at

20 degrees Celsius and changing the wavelength between 1.875 μm and 1.865 μm. Finally, temperature optimization was studied by holding the wavelength at 1.875 μm, the repetition rate at 1 Hz, and the pulse duration at 3 ms, and changing the temperature from 0, 20, or 38 degrees Celsius. Using at least 3 healthy BN3 for each pulse duration, wavelength, and temperature level, the stimulation thresholds for each optimization experiment were determined using the same protocol outlined for the repetition rate study in 3 – 6 locations (n = 12 – 16). Temperature was equilibrated to 0 Celsius using an ice bath, 38 Celsius using a hot plate, or room temperature. The temperature was recorded in these studies using a FLIR infrared camera to ensure temperature was maintained at the appropriate level for the duration of the experiment (FLIR Systems, Inc, Boston, MA).

Statistical analysis was performed for each parameter. The significance between the stimulation threshold values were calculated using a one way ANOVA analysis. Predictive values (p) less than 0.05 were considered statistically significant.

Inducing Behavioral Patterns

Studies were done to show the feasibility of inducing behavioral patterns in the neural networks of the *Aplysia*. In these studies, the neural network, comprised of the buccal ganglion and the cerebral ganglion, was extracted from the animal. In some experiments, the buccal mass was attached so that the nerves enervated the muscle. The buccal mass was suspended in a clear plastic container of saline and the two ganglia were pinned to a saline filled dish. Platinum hook electrodes were attached to buccal nerve 3 (BN3), buccal nerve 2 (BN2), radula nerve (RN), and I2 nerve. A video camera was positioned outside the container to record the movement of the buccal mass during

stimulation. An extracellular glass electrode was positioned over the cerebral-buccal interneuron on the cerebral ganglion. A 200 µm core flat polished optical fiber (Ocean Optics, Dunedin, FL) was positioned over the interneuron with a thinned layer of sheath covering it. At an 1.875 µm wavelength, 3 ms pulse duration, and 16 Hz repetition rate, the laser was systematically dialed up until it induced muscle contractions in the buccal mass. Nerve activity was recorded using data acquisition software (AxographX, Sydney, Australia). In order to find the location of the cerebral buccal inteneuron that projects from the cerebral ganglion to the buccal ganglion, a set of experiments were done in the isolated cerebral and buccal ganglia without the muscle mass attached. The nerves were clipped from the buccal mass with suction electrodes attached to the ends of I2, RN, BN2, and BN3. The laser was moved across the surface area of the cerebral ganglion and stimulated starting at a repetition rate of 13 Hz and dialing up the current to maximum output. If no stimulation was seen, the repetition rate was increased in 1 Hz increments until reaching 16 Hz. If still no neural patterns were induced, the optical fiber was moved to another position and the process continued. Activation of behavioral patterns was determined by the duration and timing of action potentials from each nerve. Distinct patterns could be identified as either ingestive or egestive feeding behavior.

Results

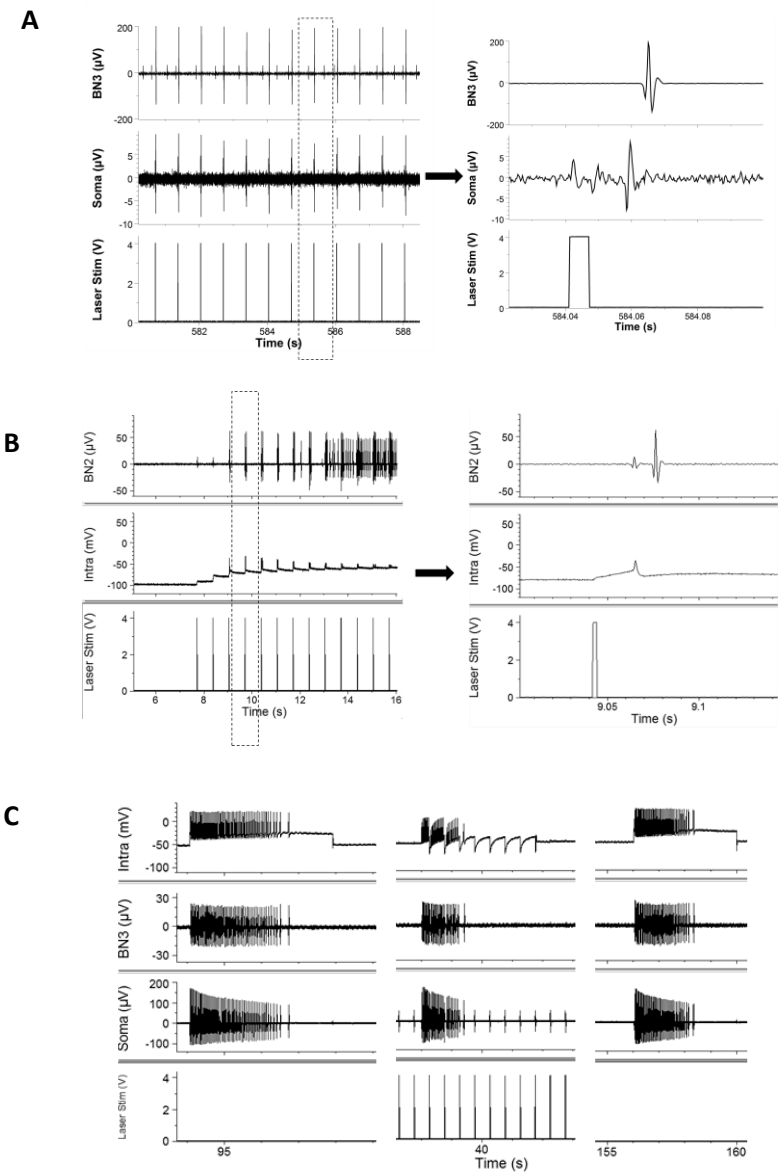

Figure 1: Infrared stimulation evokes neural spike activity in BN3 and the soma of the *Aplysia* buccal ganglion as seen in the extracellular and intracellular recordings. A: Recorded extracellular potentials from the soma and nerve are firing one to one with the laser pulses. The trace is zoomed in on the right. Laser parameters: 10.90 J/cm^2, 1.5 Hz, 200 μm spot size, 6 msec pulse duration, 1.875 μm light. B: Intracellular recordings show evoked action potentials that eventually propagate to the nerve. A zoomed in trace shows action potential response to a single laser pulse. Cell eventually dies due to high pulse energies. Laser parameters: 18.51 J/cm^2, 2 Hz, 200 μm spot size, 2 ms pulse duration, 1.875 μm wavelength. C: INS is also able to inhibit neurons that were firing due to the application of electrical current. Stimulation parameters: 17.35 J/cm^2, 2 Hz, 2 msec pulse duration, 20 nA current.

The initial feasibility of infrared neural stimulation was established using 1.875 μm light at 1.5 Hz with a spot size of 200 μm. Figure 1A represents the stimulation of the soma in the buccal ganglion while recording the responses at the soma and BN2. The signal induced by the infrared light was frequency locked with the laser repetition rate. A zoomed view of the INS stimulation in Figure 1A reveals no stimulation artifact that commonly occurs during electrical stimulation. Figure 1B shows that immediately after the onset of the laser pulse, the cell begins to depolarize intracellularly before the action potential propagates down the nerve. Because the collagenous sheath overlaying the nerve cells was removed in this preparation, the laser radiant exposure levels were much higher than stimulation threshold. Because the sheath absorbs the energy of the laser, the stimulation threshold for removed sheath preparations is much lower. Such high energy levels induced excessive activity in the cells and eventual cell death.

In addition to optically stimulating neurons, we have shown the feasibility of optically inhibiting neurons using INS. A different nerve cell was targeted than those previously shown to be stimulated by INS. Figure 1C shows intracellular nerve cell recording taken while 20 nA of electrical current was injected into the cell. During these periods of electrical stimulation, INS was applied to the same cell. In this particular neuron, the INS inhibited nerve cell firing by hyperpolarizing the cell. To ensure that the laser firing had not killed the cell, the electrical stimulus was applied again without INS, and the action potentials continued uninhibited.

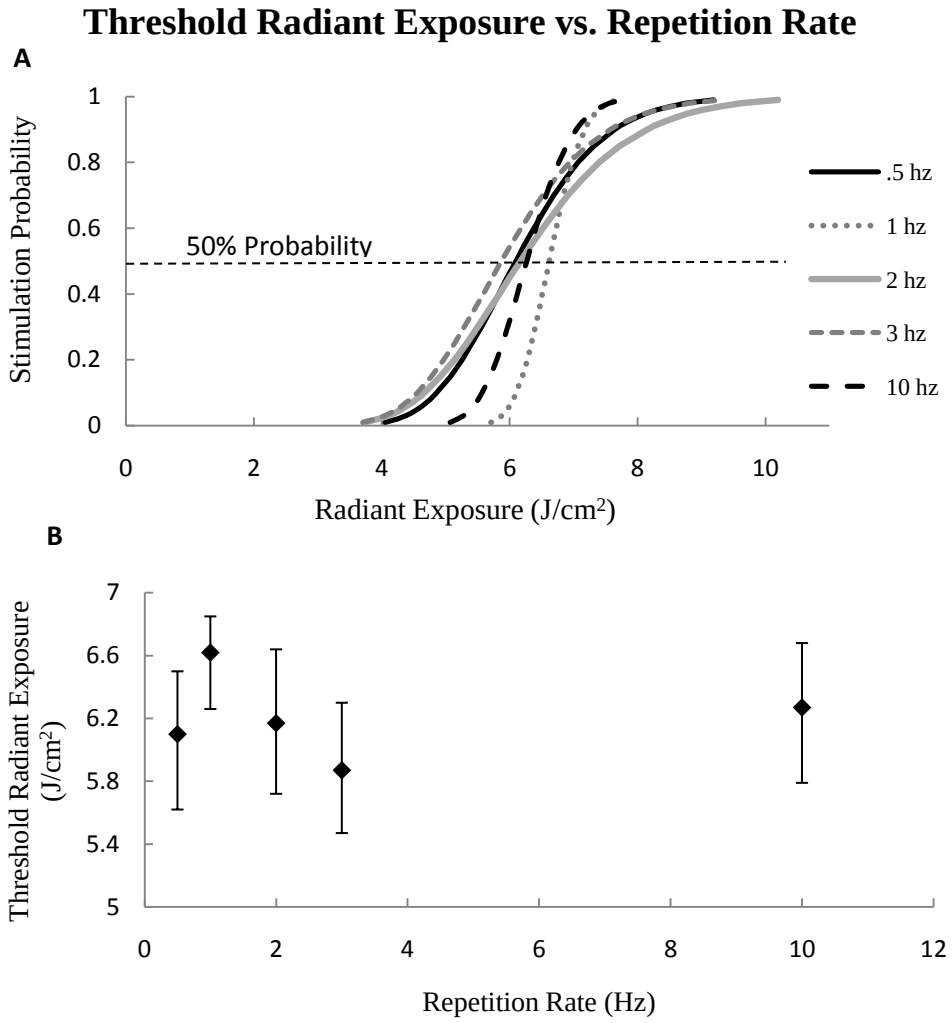

Figure 2: Infrared neural stimulation was performed at laser repetition rates of 0.5, 1, 2, 3, and 10 Hz. Top trace shows Probit regression curves which plot the stimulation probability as a function of radiant exposure level for each repetition rate. The stimulation threshold is determined to be the radiant exposure level that yields a stimulation probability of 50%. The bottom trace shows a plot of stimulation thresholds for each repetition rate level and their 90% and 10% fiducial limit. Results show no significant difference in stimulation thresholds for stimulation at all five repetition rates.

Repetition rate studies were performed using a 1.875 μm wavelength, 3 ms pulse duration with a 200 μm core diameter fiber optic at repetition rates of 0.5, 1, 2, 3, and 10 Hz. Figure 2A shows the five curves that were produced from the PROBIT analysis software pack at these repetition rates. Each probability curve represents the calculated

probability that stimulation will occur at a given radiant exposure level. The stimulation threshold, or ED_{50} value, for each curve is the radiant exposure that gives 50% stimulation probability. The data for 0.5, 1, and 2 Hz show more spread than the data for 3 and 10 Hz. This is demonstrated by the smaller slope of the probability curves for those repetition rates. There is a natural spread of the data for all Probit curves in Figure 2A due to the inherent biological variability of the *Aplysia* neural tissue.

The threshold point was taken from each set of curves and plotted along with the 90% and 10% fiducial limits in Figure 2B. From this figure, the results of the repetition rate can be summarized. The figure shows that the stimulation threshold radiant exposure values are not significantly different among for all five repetition rates tested. Furthermore, a single factor analysis of variance (ANOVA) test was performed on the stimulation thresholds for all five repetition rates. This test gave a p-value of 0.737. At a 95% confidence level, this result shows that there is no statistically significant difference between the stimulation thresholds for each repetition rate tested. The average threshold radiant exposure for all repetition rates is 6.206 J/cm^2.

Repetition rates higher than 10 Hz were also tested. Stimulation at repetition rates as high as 15 Hz and 30 Hz consistently showed action potential dropout during the twenty pulse train, even at very high radiant exposures. Although stimulation was consistent for every other or every third laser pulse, stimulation could not be measured by the same criteria as the other repetition rates.

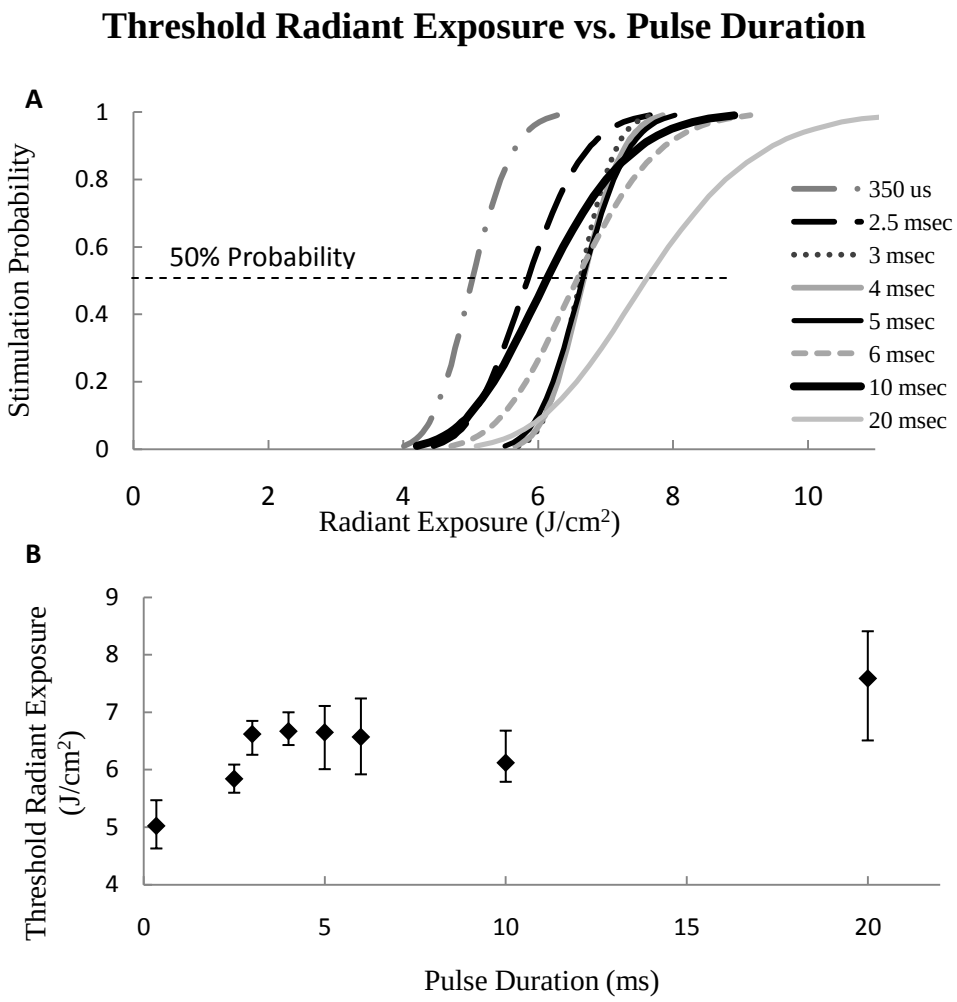

Figure 3: Infrared neural stimulation was performed at laser pulse durations of 350 µs, 2.5, 3, 4, 5, 6, 10 and 20 ms. Top trace shows probit regression curves which plot the stimulation probability as a function of radiant exposure level for each repetition rate. The bottom trace shows a plot of stimulation thresholds for each pulse duration level and their 90% and 10% fiducial limits. Results show no significant difference in stimulation thresholds for stimulation at pulse durations between 3 and 10 ms.

A pulse duration study was performed using a 1.875 µm wavelength, 3 ms pulse duration with a 200 µm core flat polished fiber optic at pulse durations of 2.5, 3, 4, 5, 6, 10, and 20 ms. A pulse duration of 350 µs was also tested using the Ho:YAG laser with a wavelength of 2.1 µm and a repetition rate of 2 Hz. In the same fashion as the repetition rate study, eight curves are produced from the PROBIT analysis software pack for each

pulse duration as shown in Figure 3A. While all eight curves reflect a natural spread in the data, the 20 ms curve shows a much larger variance due to the longer time course for stimulation.

The ED50 values for pulse durations of 0.35, 2.5, 3, 4, 5, 6, 10, and 20 ms are shown in Figure 3B. It can be seen from this figure that the ED50 values for pulse durations between 3 – 10 ms are not significantly different as shown by their overlapping standard error bars. Figure 3B demonstrates that as the pulse duration decreases below 3 ms, the threshold radiant exposure also decreases. The threshold radiant exposure for a pulse duration of 20 ms is shown to be higher than the threshold radiant exposure for lower pulse durations. An ANOVA test was performed for all eight stimulation thresholds and gave a p-value of 0.0024. At a 95% confidence level, this result shows that there is statistically significant difference between the stimulation thresholds for at least one of the pulse durations tested. Therefore, it cannot be assumed that the stimulation thresholds are equal for all pulse durations tested. From looking at the plot shown in Figure 3B, it can be seen that the threshold radiant exposure for the 20 ms pulse duration is much higher than the threshold radiant exposures of the other pulse durations. The average threshold radiant exposure for pulse durations between 3 to 10 ms is 6.526 J/cm^2, while the threshold radiant exposure for stimulation at 20 ms was determined to be 7.59 J/cm^2 and the threshold radiant exposure at 350 μs was 5.02 J/cm^2.

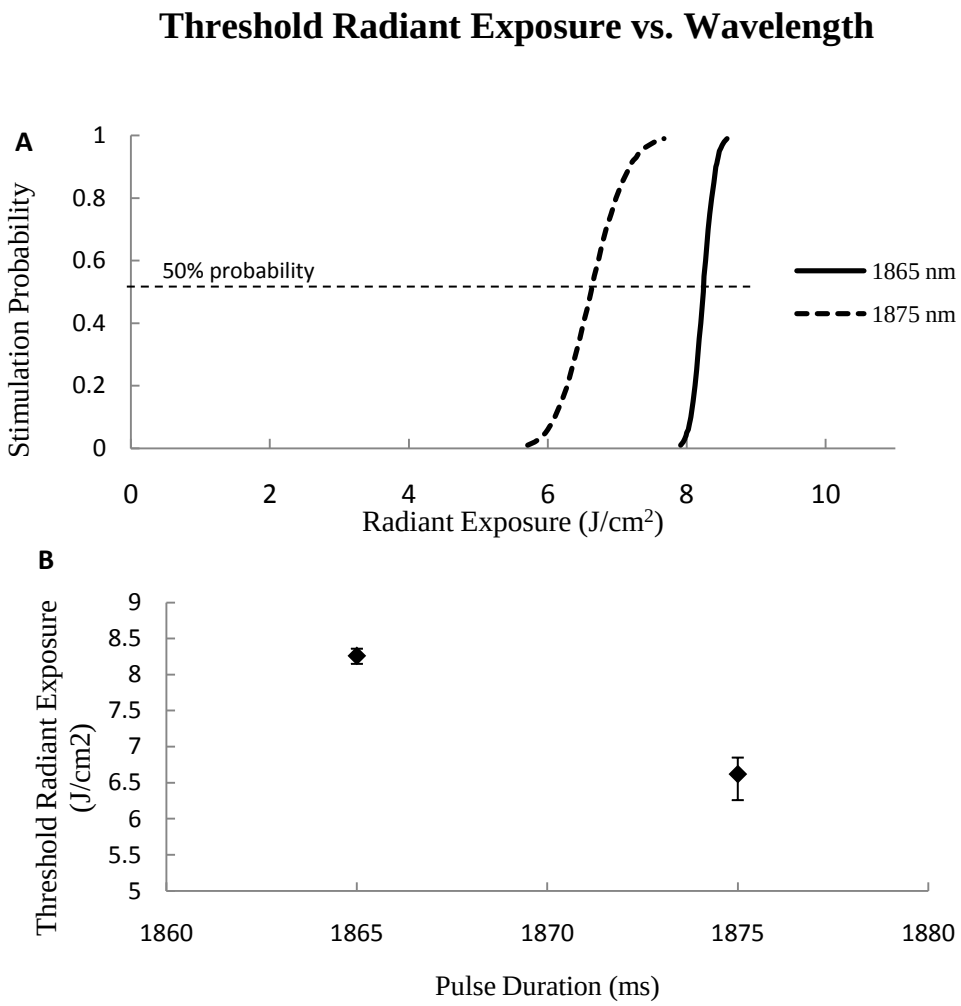

Figure 4: Infrared neural stimulation was performed at wavelengths of 1.865 µm and 1.875 µm. Top trace shows Probit regression curves which plot the stimulation probability as a function of radiant exposure level for each wavelength. The bottom trace shows a plot of stimulation thresholds for each stimulation wavelength and their 90% and 10% fiducial limit. Results show that stimulating with 1.865 µm light requires significantly more energy to induce action potentials than stimulation with 1.875 µm light.

The wavelength dependence was established using a 2 Hz repetition rate, 3 ms pulse duration with a 200 µm core flat polished fiber optic at two wavelengths of 1.865 and 1.875 µm. In the same fashion as the repetition rate study, two curves are produced from the PROBIT analysis software pack for each wavelength as shown in Figure 4A.

The ED50 values for wavelengths of 1.865 and 1.875 µm are shown in Figure 4B. Both wavelengths successfully stimulated BN3, but the optimal wavelength with minimal radiant exposure was identified to be 1.875 µm. The average radiant exposure at 1.875 µm was 6.62 J/cm^2 and 8.26 J/cm^2 at 1.865 µm.

A two sample t-test was performed and produced a p-value of 2.399e-5. At a 95% confidence level, this result shows that there is a statistically significant difference between the stimulation thresholds for each wavelength. Stimulation at wavelengths below 1.865 µm were tested but yielded no stimulation.

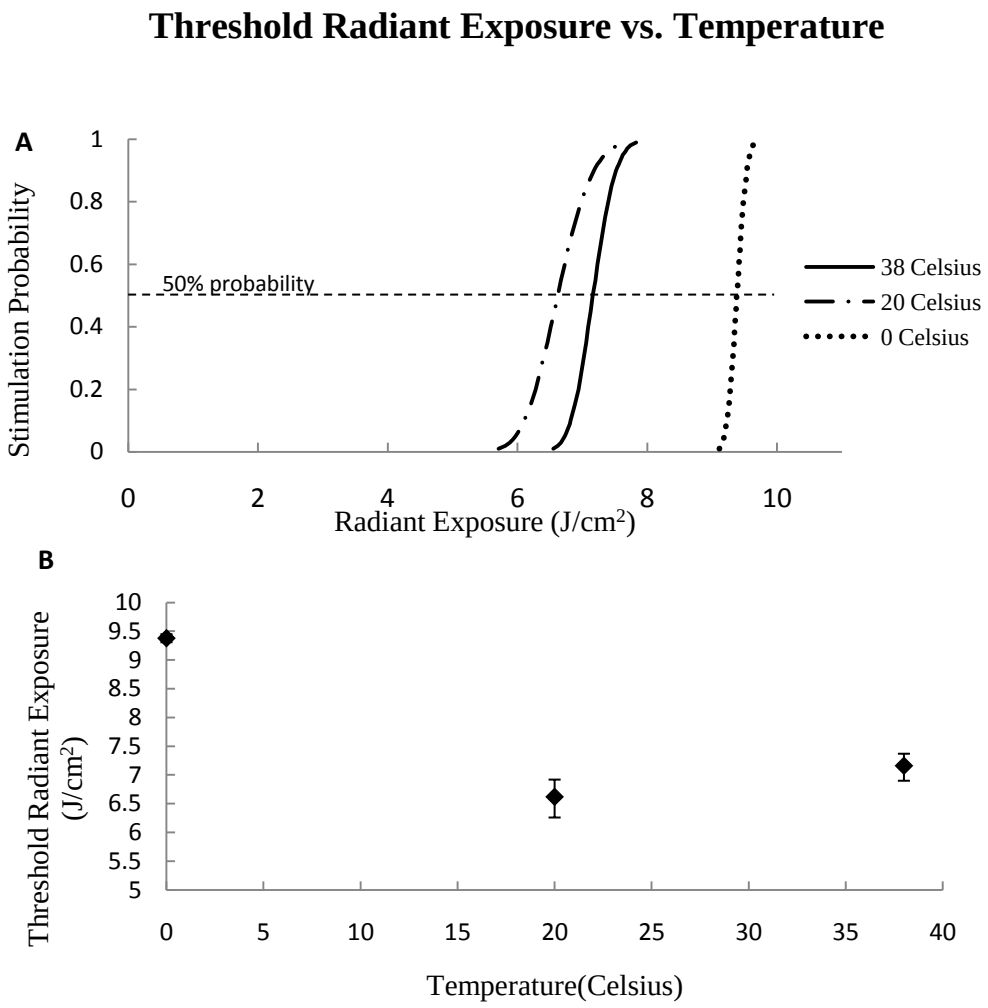

Figure 5: Infrared neural stimulation was performed at temperatures of 0, 20 and 38 Celsius. Top trace shows Probit regression curves which plot the stimulation probability as a function of radiant exposure level for each temperature. The bottom trace shows a plot of stimulation thresholds for each temperature and their 90% and 10% fiducial limit. Results show that stimulating at an ambient temperature of 0 Celsius requires significantly more energy to induce action potentials than stimulation at 20 or 38 Celsius.

The temperature dependence was established using a 2 Hz repetition rate, 3 ms pulse duration with a 200 μm core flat polished fiber optic at three ambient temperatures of 0, 20 and 38 Celsius. In the same fashion as the repetition rate study, three curves are produced from the PROBIT analysis software pack for each ambient temperature as

shown in Figure 5A. The ED50 values for each temperature and their 10% and 90% fiducial limits are shown in Figure 5B. The stimulation threshold when the ganglion was in a 0 C bath was significantly higher than at 20 and 38 C. The average radiant exposure at a temperature of 0, 20 and 38 C was 9.38 J/cm^2, 6.62 J/cm^2 and 7.16 J/cm^2, respectively. A two sample t-test was performed and produced a p-value of 2.88e-8. At a 95% confidence level, this result shows that at least one of the stimulation thresholds is significantly different.

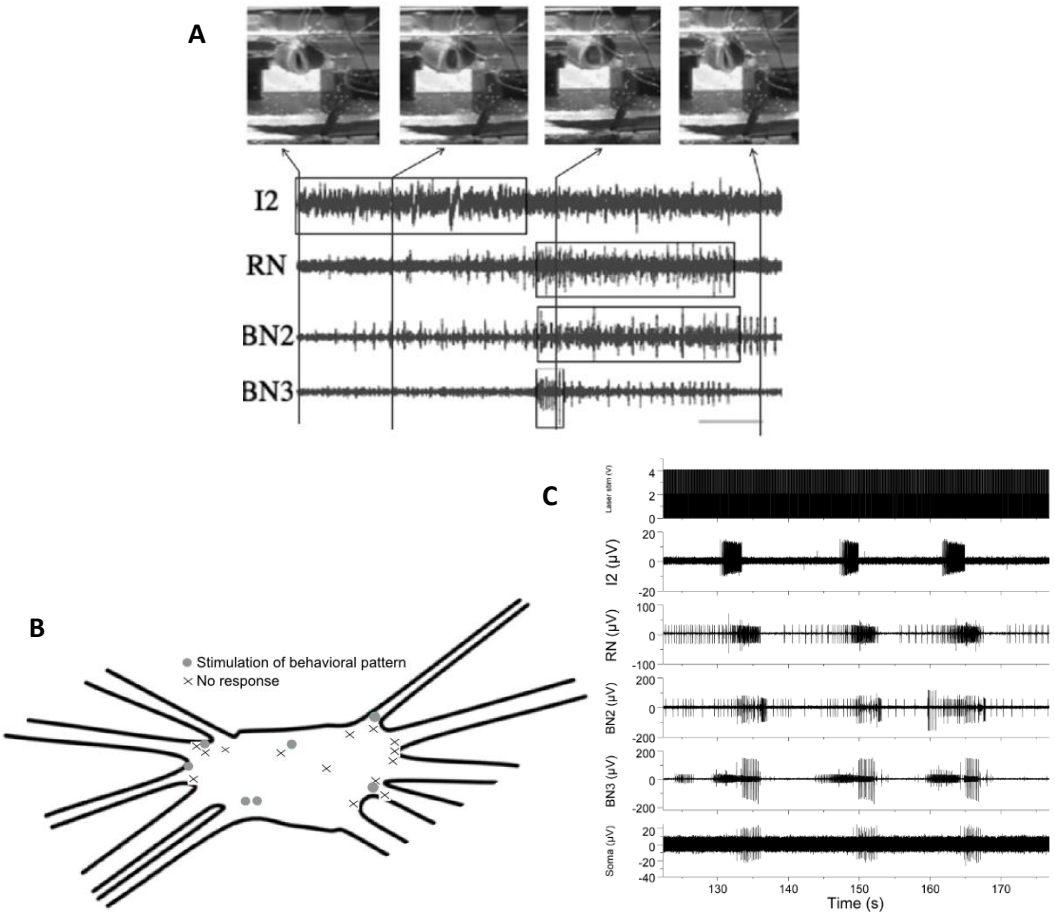

Figure 5: Infrared stimulation evokes ingestive behavioral patterns in the neural networks of the *Aplysia*. A: Neural spike activity in extracellular recordings corresponds to opening and closing of the buccal mass. B-C: Locations of cerebral buccal interneurons on the cerebral ganglion that induced patterns were determined. The ingestive behavioral patterns induced are shown in C. Stimulation parameters: 6.62 J/cm^2, 16 Hz, 3 ms pulse duration.

The feasibility of inducing complex behavioral patterns in the neural networks of *Aplysia* was established using 1.875 μm at 16 Hz, 3 msec, and a spot size of 200 μm. Figure 6A represents snippets from a video recording of the buccal mass during stimulation of the neural network comprised of the buccal and cerebral ganglion. Stimulation of a cerebral buccal interneuron on the cerebral ganglion induced neural spike activity on BN3, BN2, RN, and I2 nerves. The pattern of stimulation is recognized

as ingestive behavior due to the timing of the activity on each nerve. A video recording of the suspended buccal mass attached to the nerves showed a contraction and relaxation of the buccal mass mimicking the ingestion of food in an intact animal. A repetition rate as high as 16 Hz and a radiant exposure of 6.62 J/cm^2 was required to induce this behavior.

Initial studies showed that the stimulation of a single neuron could induce complex patterns, and further studies were done to determine the locations of neurons that can induce this behavior. Figure 6B shows the location of the neurons that induce ingestive behavior when optically stimulated at repetition rates between 10 – 13 Hz, a radiant exposure between 6.33 – 9.42 J/cm^2, and a pulse duration of 3 msec. The patterns induced were repeatable as shown in Figure 6C.

Discussion

This study presents the characterization of INS in an invertebrate model organism in order that the scientific community may eventually garner a more full understanding of the physiological mechanism and optimal laser parameters for INS. Studies done by Wells et al. reported neural activation with pulsed infrared light is caused by a thermal transient in the tissue. The mechanism by which heat caused neural activation was thought to be from the direct or indirect activation of transmembrane ion channels (J. Wells et al., 2007c). Fork reported an increase in action potential firing frequency when stimulating spontaneously firing nerve cells in *Aplysia* using continuous wave visible laser light (R.L Fork, 1971). The results of this study show the ability to stimulate or in some cases inhibit nerve cells in *Aplysia californica* in a way that is spatially precise and artifact-free using pulsed infrared light (Figure 1). These results show that stimulation is

possible in the neurons of *Aplysia* through the collagenous sheath at high levels of power. At a wavelength of 1.875 μm, the infrared light penetrates 333 μm into the tissue according to the water absorption spectra (J. Wells et al., 2007c). Higher radiant exposure levels are necessary to penetrate the collagenous sheath because the water in the sheath absorbs optical energy before it can reach the nerve cells. Direct stimulation of action potentials on the soma and nerve were shown in a one-to-one fashion, frequency locked with each laser pulse. Intracellular depolarization occurs immediately after the onset of the laser pulse, which can be seen from the experiments done on the neurons without the sheath. This intracellular depolarization accounts for the delay between the laser pulse and the extracellular action potential. An optimal radiant exposure level must be found because laser energy levels far above threshold induced excessive neural activity before causing cell death. The inhibition of nerve cells was also shown in these experiments to be repeatable. By increasing the baseline voltage and applying INS, it is apparent that each cell has a reversal potential that it tends toward when stimulated. The reversal potential of the nerve cells is a result of the contribution of the opening of several ion channels, which are not known. Knowledge of the ion channels responsible for stimulation and inhibition would provide an understanding of the physiological mechanism of INS and provide a means to controlling neural behavior in intact, behaving organisms.

Repetition rate is an important parameter to consider for the optimization of INS. Previous studies done by Wells et al. show that no thermal damage was observed by stimulating at 2 Hz, but repetition rates of 5 and 8 Hz shows the possibility of some thermal damage to the nerve (J. Wells et al., 2007b). The results presented here (Figure 2)

show that stimulation of the nerve was repeatable and produced frequency locked action potentials for repetition rates ranging from 0.5 – 10 Hz. This result is unexpected in light of previous studies that show temperature superposition occurring in the first few pulses when stimulating at high repetition rates. The results indicate that there is no change in the threshold radiant exposure needed for nerve stimulation within the range of repetition rates tested. It is expected that the threshold radiant exposure would not change for repetition rates lower than 0.5 Hz because the time between laser pulses is sufficient for thermal dissipation.

At repetition rates higher than 10 Hz, action potential drop out occurred, yielding a neural response that was not one-to-one with the laser pulse. The inability to induce one-to-one action potentials for each laser pulse stimulating BN3 at repetition rates of 15 and 30 Hz is unusual in relation to the findings of other research groups in experiments done in mammalian models. Consistent firing has been reported at repetition rates on the order of hundreds of Hertz (A.D. Izzo et al., 2007). The action potential dropout observed may be accounted for in several ways. The dropout may suggest the activation of a slow process that limits consistent stimulation at higher repetition rates. When action potentials are stimulated in close temporal proximity, potassium ions build up outside of the cell. This enhances sodium channel inactivation, which prevents the flow of sodium ions for a few milliseconds after depolarization. Our results from stimulation at repetition rates over 10 Hz suggest that there is a time constant that must be taken into account to yield consistent action potential firing for every laser pulse. This time constant may be attributed to the longer duration of *Aplysia* action potentials, which can range from 12 – 20 ms. The longer duration is about twice that of many mammalian motor neuron action

potentials, and it corresponds to a longer refractory period. Additionally, *Aplysia* function optimally in temperatures around 16 Celsius. At this temperature, many activation processes will be slower than at the standard body temperatures in mammals, which is around 37 Celsius. It is also important to note that *Aplysia* nerves are unmyelinated, which means that action potentials cannot propagate as fast as mammalian nerves that utilize saltatory conduction. The slower propagation of action potentials may prevent *Aplysia* from firing consistently at higher repetition rates.

Pulse duration is another important parameter for the optimization and understanding of INS. Several studies have been done to understand the effects of pulse duration on threshold radiant exposure. Work done by Izzo et al. showed that threshold radiant exposure increased with increasing pulse durations ranging from 35 μs – 1 ms in the gerbil cochlea (A.D. Izzo et al., 2007). Wells et al. showed that at higher pulse durations ranging three orders of magnitude from 5 μs – 5 ms threshold radiant exposure levels were not significantly different in the rat sciatic nerve (J. Wells et al., 2007c). The work presented here shows an alternative trend for the pulse durations tested as compared to previous studies on other pulse durations. The threshold radiant exposure was constant in the range of 3 – 10 ms, but it increased above and decreased below that range (Figure 3B). A higher threshold radiant exposure when stimulating at a 20 ms pulse duration is consistent with the thermally mediated mechanism of INS proposed by Wells (J. Wells et al., 2007c). The thermally mediated mechanism can be explained by understanding that the laser energy is absorbed by the tissue and converted to heat. The data presented indicate that the heat is spatially and temporally confined for pulse durations shorter than 10 ms. For soft tissue, the thermal relaxation time is approximately 90 ms (J. Wells et al.,

2005b; J. Wells et al., 2007c). During the 20 ms pulse duration, a significant amount of thermal energy begins to dissipate away from the site of absorption before the full pulse has been delivered to the tissue. Therefore, a higher threshold is required to overcome the heat loss during dissipation because energy deposited after ~10 ms does not significantly contribute to the generation of an action potential. The lower threshold needed for stimulation using pulse durations below 3 ms indicates that it is not the total thermal rise that is responsible for optical stimulation. The data presented here indicate that the shorter the pulse duration, the less energy required for stimulation. Therefore, the time over which the energy for stimulation is deposited governs optical stimulation. Stimulation at 350 μs may require less energy than stimulation at 2.5 μs because the time course for stimulation more efficiently induces the processes that cause neural activation. It is important to note that the laser required to stimulate at 350 μs used a wavelength of 2.1 μm, which is higher than the wavelength used for the other pulse durations. Because this wavelength will fall at a different location on the water absorption spectrum and yield a different penetration depth, confounding factors may play a role in its lower stimulation threshold.

Previous studies have shown that wavelength is a significant parameter in INS because it determines the penetration depth of the optical energy in tissue (J. Wells et al., 2005a; J. Wells et al., 2005b; J. Wells et al., 2007a). The peripheral nerve tissue is ~80% water, which means that the water is the primary absorber of optical energy (H.F. Rosenberg et al., 1959). According to the water absorption curve shown in Figure 7, the absorption coefficient (cm^{-1}) is a function of the wavelength of light used in INS. The absorption coefficient is inversely proportional to the effective penetration depth, which

is defined as the depth in the medium at which the radiant exposure is reduced to 1/e times (~37%) the incident irradiance (J. Wells et al., 2007a). Therefore, wavelengths of light that correspond to higher absorption coefficients on the water absorption curve yield lower effective penetration depths, and lower absorption coefficients yield higher effective penetration depths. The results presented here (Figure 4) show that stimulation at a wavelength of 1.875 µm is a more efficient wavelength of light for stimulation of the *Aplysia* nerve than a wavelength of 1.865 µm. The effective penetration depth at 1.875 µm is 400 µm whereas the effective penetration depth at 1.965 µm is 625 µm. Stimulation at 1.875 µm allows for a more optimal deposition of energy within the neural tissue because it minimizes the amount of energy deposited in the non-excitable layer of collagenous sheath surrounding the outside of the axon bundle. Attempts to stimulate at wavelengths below 1.865 µm yielded no neural response, indicating that optical energy that penetrates deeper than 625 µm is deposited outside of the nerve bundle and does not contribute to stimulation.

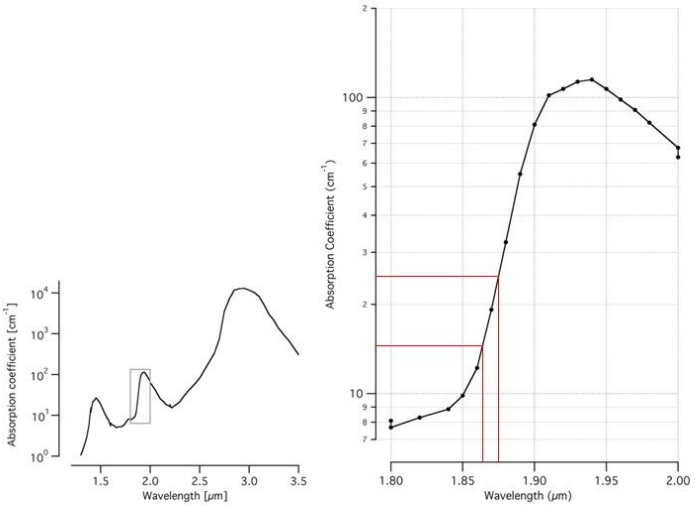

Figure 7: Water absorption curve. The absorption coefficients for wavelengths of 1.875 µm and 1.865 µm are 25 cm^{-1} and 16 cm^{-1}, respectively. These absorption coefficients correspond to effective penetration depths of 400 and 625 µm (AD Izzo et al. 2008).

The last parameter tested was temperature. Previous studies done by Wells et al. showed that the mechanism of INS was thermally mediated (J. Wells et alk., 2007c). The photothermal effects that cause INS are governed by the absorption of optical energy in the neural tissue and its conversion to heat energy which leads to a local energy increase. The nature of the temperature increase can be due to a time dependent thermal gradient or as a result of reaching a minimum absolute temperature at the level of the axon bundle. Experiments done in the rat sciatic nerve showed that there was no significant difference in the threshold radiant exposure at 0 and 25 Celsius. This pointed to a thermal gradient as the nature of the photothermally mediated mechanism (J. Wells et al., 2007c). The results presented here also show that raising the ambient temperature from 0 to 20 Celsius does not cause a significant change in threshold, but changes in the temperature as high as 38 Celsius does cause a significant decrease in radiant exposure needed for stimulation in *Aplysia* (Figure 5). The environment of this invertebrate typically ranges from 14 – 25 Celsius, but studies have shown that its behaviors such as egg-laying are temperature dependent. In warmer temperatures, egg laying is facilitated, and it is attenuated at colder temperatures (N.L. Wayne et al., 1996). It was also noted that while the temperature was raised as high as 38 Celsius, the spontaneous firing of the nerve was much more frequent. The increase in ambient temperature may decrease the threshold radiant exposure and affect action potential propagation in several ways. One cause may be that the Nernst equilibrium potentials are inversely proportional to the absolute temperature. Another hypothesis is that the conductance of open ion channels depends on the Q10, or the common temperature factor that influences the rate of channel induction. Further studies must be done to understand exactly how the temperature change affects action potential

propagation in the *Aplysia*. However, these results indicate that absolute temperature increase cannot be eliminated as an explanation for the thermally mediated mechanism.

Optically induced behavioral patterns in the neural networks of the *Aplysia* is one of the most exciting and applicable findings for INS community. The result presented here show one of the first reports of a single optical stimulus causing complex and meaningful neural activity that was seen to translate into muscle movement. Ingestive and egestive behaviors were observed through stimulation of a cerebral buccal interneuron in a manner that was replicated through electrical stimulation of the same neural network (Figure 6). This finding has significant implications for the future of implantable neural prostheses that utilize INS. In a minimally invasive, artifact free, spatially specific manner, these results show that INS may one day be able to induce fully functioning motor movement in humans with lost functioning. The results presented here also show the characterization of the location of the cerebral buccal interneuron on the cerebral ganglion that can be stimulated to induce these behaviors. This mapping of the ganglia provides the groundwork of future studies that can further characterize the behavioral patterns in *Aplysia* induced by INS. The laser parameters necessary to induce these patterns requires higher repetition rates than is necessary for single nerve stimulation. Higher repetition rates may be causing damage because activation was seen to diminish after several attempts at stimulation. These laser parameters must be further characterized in order to ensure safe stimulation in the future.

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CHAPTER III

Future Directions

Infrared neural stimulation (INS) has been performed in the ganglia of *Aplysia californica ex vivo*. Neural activity was measured from neurons and nerves in the buccal ganglion and cerebral ganglion using electrophysiology techniques. The results presented in this study show that INS is feasible in this invertebrate model, making it a viable candidate for further studies of the physiological mechanism and optimal laser parameters of INS. Parameterization studies show threshold radiant exposure does not change significantly across repetition rates ranging from 0.5 – 10 Hz. Threshold radiant exposure, however, does not hold constant for all pulse durations. A wavelength of 1.875 μm was shown to be more optimal than lower wavelengths tested because of its ideal penetration depth of 400 μm, which successfully penetrates the layer of collagenous sheath covering the nerve without letting light leak outside the tissue. Temperatures above 20 Celsius were shown to have an effect on stimulation threshold while lower temperatures did not. Behavioral studies showed that stimulating neural networks of the *Aplysia* could induce meaningful and complex behavioral patterns. The results from this study will allow the further investigation of the optimal laser parameters and a deeper look into the physiological mechanism of INS.

INS as a whole is aimed at providing an alternative stimulation modality to be used clinically in neural prostheses and implants as well as in the laboratory to study neural connectivity. This project on INS in *Aplysia* is directed at furthering the knowledge of the biomedical community on how INS works and can best be

implemented. The opening of ligand gated channels is a possible mechanism for action potential propagation via INS. Temperature change may activate sodium, potassium, or chloride ion channels by increasing their conductance and transitioning to an open state. Altering the ion content of the saline bathing the buccal ganglion during stimulation will reveal how each ion affects stimulation. A detailed study of how increasing or decreasing sodium or other ion concentration affects threshold radiant exposure will provide insight into the leading factors responsible for stimulation. The mechanism may not have to do with ligand gated channels, but instead be due to the activation of temperature sensitive channels. It is unknown whether temperature sensitive channels, such as TRP channels, exist in the bucccal ganglion of the *Aplysia*. Performing histology to determine the presence of certain types of temperature sensitive channels will provide direction. If they do not exist, INS is caused by some other mechanism. If they do exist, channel blockers can be applied to observe changes in stimulation threshold. Gaining an understanding of the ion channels directly or indirectly responsible for action potential induction using INS will allow the creation of devices that can more easily manipulate neural activity.

The use of INS to induce complex behavior is one of the most promising directions for the study of *Aplysia.* The knowledge that single point stimulation can yield a meaningful behavioral pattern has significant implications for the future of clinically used devices for patients with paralysis and limb loss. Current prosthetic devices allow very course movement and do not fully replicate the complex range of motion that normal functioning limbs allow. Further studies in the neural networks of the *Aplysia* will provide a better understanding of the interconnectedness of neural circuitry that leads to complex patterns of movement. Before performing a detailed study of these movements

in the *Aplysia*, it is important to quantify bite intensity. A quantification of buccal mass movement will allow parameter optimization studies. The results of these studies will be used to determine if and why higher repetition rates are required to stimulate patterns of behavior. Further studies will be done on vertebrate animals to determine if similar behavioral patterns can be induced in more complex systems. Eventually these findings may be used to develop an implantable device either in the peripheral nerve or in the central nervous system to yield meaningful behavior in a spatially precise manner using INS.