A BEHAVIORAL ANALYSIS OF HABITUATION WITHIN SIMPLE INVERTEBRATE NERVOUS SYSTEMS

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A Behavioral Analysis of Habituation within Simple Invertebrate Nervous Systems

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Abstract

Lockery, Rawlins, and Gray's (1985) study indicated that (a) habituation and dishabituation were elicited in the shortening reflex of the medicinal leech by photic and electrical stimulation, and that (b) the neural mechanisms that underlie behavioral responses, parallels behaviors of the gill-withdrawal reflex in *Aplysia californica*. However, Kristan et al. (1982) and Debski and Friesen's (1985) studies suggest that a swimming response can be elicited by electrical stimulation to the specific region of the leech. Similar to studies of *Aplysia californica*, the leech demonstrates nonassociative learning, including habituation and sensitization, through a variety of behavioral responses. Thus, the present study replicated Kristan, McGirr, and Simpson's (1982) and Debski and Friesen's (1985) research, to demonstrate (a) habituation and (b) a swimming response as a result of electrical stimulation to the posterior area of the leech. As expected, a swimming response was elicited when stimulus were presented to the posterior region. However, habituation did not occur.

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Within the field of psychology, habituation is perhaps the simplest form of learning. This term refers to the decline of an organism's tendency to respond to a stimulus once the stimulus becomes familiar; in other words, an organism learns to ignore a weak stimulus that is repeatedly presented when the consequences of the stimulus are neither noxious nor rewarding (Hawkins & Kandel, 1984.) Habituation, which relies on memory, forces an organism to compare what it currently hears and sees, with what it has previously heard and seen; although the organism learns to recognize an event as familiar, it does not learn anything new about that event. For this reason, one of the benefits of habituation is that it narrows the range of stimuli that elicits a response.

Habituation

The importance of habituation in learning has been emphasized by a common problem researchers have examined over the past two decades. This problem focuses on how different forms of learning are related to one another and the mechanisms that govern them. Specifically, the challenge presents two distinct possibilities: each form of learning may be governed by a fundamentally different mechanism or they may be governed by variations on a common mechanism (Hawkins & Kandel, 1984.) Answers to these possibilities are crucial to researchers as it yields insight at the molecular and behavioral levels of neural plasticity. Moreover, answers would allow researchers to determine whether or not mechanisms of higher forms of learning may similarly be based on the mechanisms of these simple forms of learning.

Learning

Learning has traditionally been divided into two distinct forms: associative learning and nonassociative learning. Associative learning includes classical and operant or instrumental conditioning, while nonassociative learning includes habituation and sensitization.

In classical conditioning, a reflexive response is acquired after a neutral stimulus, the conditioned stimulus (CS) is paired with an unconditioned stimulus (US), regardless of what the organism does. This was most clearly demonstrated by Pavlov's study of salivation in dogs. Within his studies, Pavlov found that the salivary reflex could be set off by a range of other stimuli, besides the sight or smell of food. To study learning, Pavlov repeatedly sounded a bell, followed by the presentation of food; then he observed what happened when the bell was sounded alone, without any food. The result was clear: the repeated pairing of the bell with food led to salivation even when the bell was presented by itself; a wide range of stimuli, but not all, respond to conditioning.

Operant conditioning differs from classical conditioning in a number of ways. The most important is that, in operant conditioning, reinforcement depends upon the proper response. Another difference concerns the selection of the response. In operant learning, the response must be selected from a set of alternatives. Specifically, operant conditioning makes use of a reinforcer, which is given only if the organism performs the instrumental response; in effect, what has to be learned is the relationship between the response and the reinforcer.

The experimental study of operant learning was made possible by a method devised by Thorndike. His method employed the use of a puzzle box; a hungry animal was placed inside the box, which could be easily opened by performing some simple action. Once outside the puzzle box, the animal was rewarded. By carrying out numerous trials, Thorndike was able to examine operant learning in the form of the learning curve: the curve declined quite gradually as the learning process proceeded. This learning curve suggested that the animals learned to escape in small increments, with no evidence at all of understanding or insight.

Later, B.F. Skinner extended Thorndike's findings by devising a procedure in which the instrumental response could be performed repeatedly and rapidly. This was done by employing the use of an experimental chamber, or Skinner box, in which a rat pressed a lever or a pigeon pecked at a lighted key. Unlike Thorndike's puzzle box, the Skinner box allowed the animal to remain in the presence of the lever or key for an extended period of time, while choosing the rate at which it conducted the preferred action.

Habituation and sensitization are examples of non-associative learning, in which an organism learns that significant associations do not always exist between events; events may be unrelated or irrelevant. Habituation occurs when a previously-displayed response diminishes, when no reward or punishment follows; this lack of response is not a result of fatigue or sensory adaptation. When fully habituated, an organism will not respond to a stimulus even though weeks or months have elapsed since the last presentation. Sensitization occurs when the response to a harmless stimulus increases, when that stimulus occurs after a punishing stimulus. Specifically, sensitization is thought to underlie both adaptive, as well as maladaptive learning processes in the organism.

Aplysia californica

Kandel, the most notable researcher regarding the neural basis of nonassociative learning, observed the gill withdrawal of the sea hare, *Aplysia*. Kandel and his colleagues showed that after habituation from siphon touching, gill withdrawal response weakened. To restore gill withdrawal once again, a paired noxious electrical stimulus was applied to the tail and a touch to the siphon. After this sensitization, a light touch was applied to the siphon; *Aplysia* produced a strong gill withdrawal response. When tested several days after the initial trials, this response was still manifested.

Influenced by the groundbreaking work of Kandel, the *Aplysia* (sea hare), as well as *Drosophila (*genus of small flies), *Hermissenda* (opalescent sea slug), *Astacoidea* (locust), and *Acrididae* (crayfish), have been the main focus for the study of invertebrate behavioral plasticity. In particular, the *Aplysia californica* has been a useful model because of a defensive withdrawal reflex of the mantle organs of this sea hare; a large sea slug with a soft internal shell. This simple behavior is illustrated by a well-defined neural circuit, which underlies a variety of forms of nonassociative learning. The withdrawal reflex studied in *Aplysia californica* consists of the coordinated contraction of the siphon, gill, and mantle shelf in response to tactile stimulation. This reflex is similar to vertebrate defensive escape and withdrawal responses, which can be modified by experience (Hawkins & Kandel, 1984).

The siphon is a funnel-like spout that extends from the posterior region between the parapodia and is thus, externally visible (Stopfer & Carew, 1987.) The gill is quite the opposite, as it is obscured by the mantle shelf, which in turn is covered by the parapodia, preventing any external observation (Figure 1.) Due to the anatomical arrangement, siphon withdrawal is easily monitored in freely moving animals; response magnitude in this case is typically expressed in terms of the duration of the siphon withdrawal. The gill can only be reliably observed in restrained animals, where the parapodia and mantle shelf are withdrawn. Response magnitude in this case is usually expressed in terms of amplitude of gill withdrawal. The significance of the two different response measures coincides with the preparations used: siphon duration, unrestrained, freely moving animals; gill amplitude, restrained preparations. As expressed by Stopfer et al. (1970), these two distinct response measures are useful in behavioral experiments; it has generally been assumed that one component of the reflex provides a reliable index of the other.

Thus, in *Aplysia,* habituation occurs when the animal initially responds to a tactile stimulus to the siphon by quickly withdrawing its gill and siphon. In some instances, reflex responses of the animal are reduced to a fraction of their initial value with repeated exposure to a stimulus (Hawkins & Kandel, 1984.) Thus, habituation can last from minutes to weeks depending on the number and pattern of stimulations.

To assess, Pinsker et al., (1970) conducted a 3-part experiment, which tested the neural correlates and mechanisms of habituation and dishabituation of the gill withdrawal reflex in *Aplysia.*

In their first experiment, Pinsker et al., examined habituation and dishabituation as seen in the behavioral reflex, by the abdominal ganglion of *Aplysia.* This ganglion, which offers a number of advantages for cellular neurophysiology, contains a small number of nerve cells; all of which are large enough for recording synaptic potentials and for direct stimulation with microelectrodes. Similarly, due to their large size, most of these nerve cells have been identified as unique individuals or as members of functional groupings. The connections of some of the cells with each other and with peripheral sensory and motor structures have also been specified.

In order to restrain *Aplysia* with minimum damage and to apply reproducible stimuli to peripheral sensory receptors, *Aplysia* were immobilized in a seawater aquarium; gill contractions were monitored with a photocell placed under the gill. To evoke the gill withdrawal reflex, the area along the dorsal edge of the mantle shelf was pinned to allow the delivery of a brief jet of seawater.

Pinsker et al. first examined the responses of the animal to individual stimuli that were presented to the same spot on the skin and differed only in intensity. Their results suggested that the weakest stimulus evoked only a small gill contraction which consisted of a simple, short latency withdrawal. Stronger stimuli evoked bigger and longer lasting gill responses of similar short latency, but, if strong enough, these stimuli also brought in a second component that usually had a much longer latency.

To ensure the simplification of behavioral analysis of habituation, the stimulus intensity was adjusted to obtain a short-latency element in the absence of a later component.

Overall, Pinsker et al., found that the gill responses habituated to an average of 25 percent of control amplitude (5 to 45 percent) with repetition of the tactile stimulus at intervals that ranged from 30 seconds to 5 minutes. Habituation was sometimes seen with intervals as long as 20 minutes between stimuli. Typically, the first 5 to 10 stimuli in a series produced the major part of the decrement. Full recovery from habituation required periods of rest that ranged from 10 minutes to more than 2 hours. Percent recovery was plotted in 44 separate response habituations with a single stimulus given after different intervals of rest, to obtain a more quantitative measure of the rate of recovery. About 75 to 85 percent of recovery was accounted for by a rapid phase lasting 10 to 20 minutes. This was followed by a slow and highly variable return to the original response level and often beyond (Pinsker et al., 1970).

After habituation of the response, a single strong tactile stimulus was presented to another part of the animal to produce dishabituation. Dishabituation, the restoration of a previously decremented response, occurred following a change in the stimulus pattern, such as the presentation of another, stronger stimulus.

Later, Pinsker et al., (1970) extended their findings and examined the neural correlates of habituation, as well as dishabituation, of the gill-withdrawal reflex. The behavioral response of the gill was recorded by obtaining intracellular recordings from gill motor neurons in the abdominal ganglion of the semi-intact preparation. The findings indicated that habituation and dishabituation were not due to peripheral changes in either the sensory receptors or the gill muscles, but were due to changes at a cellular level. The gill motor neurons produced these changes, which occurred in the amplitude of the excitatory synaptic potentials.

For recording purposes, two types of semi-intact preparations were used to stimulate single identified motor cells, while movements of the gill were monitored. One type of preparation consisted of the headless animal, in which all of the nervous system except the abdominal ganglion was removed. The second type of preparation immobilized the animal in a small aquarium, where jets of seawater were used as stimulation. The abdominal ganglion was externalized and a small slit was made in the body wall at the posterior end of the ciliated groove; the ganglion was pinned in place with its nerves and connectives intact (Figure 2.) Additionally, gill motor neurons were pierced with double barrel microelectrodes for intracellular recording and for changing the membrane potential.

The neural circuit of the gill reflex consists of at least four motor neurons that appear to receive monosynaptic excitatory connections from mechanoreceptors in the skin. All of these motor neurons were examined and showed similar results, but more of the work was done on cell L7 (Figure 2.)

These investigations found that contractions of the gill produced by intracellular stimulation of gill motor neurons or by electrical stimulation of efferent nerves did not substantially decrease in magnitude when evoked at intervals (1 to 5 minutes) that were effective in producing habituation. Specifically, the size of a gill response produced by intracellular stimulation of gill motor neurons was the same before and during maximum habituation of the reflex. Following a dishabituatory stimulus, a directly evoked gill contraction remained unchanged, while the evoked gill contraction increased in amplitude. Thus, habituation or dishabituation of the gill reflex cannot be explained by peripheral motor factors.

Some changes in either the sensory input or its central processing must be associated with habituation, since it was not due to fatigue of the peripheral muscle. In either case, habituation would be associated with changes that would be reflected in the gill motor neurons. Recording from the major motor neurons of the gill reflex could test this inference. Thus, Pinsker et al. (1970), found that large excitatory postsynaptic potentials (EPSPs) were produced in all the identified motor neurons of the gill, due to tactile stimulation within the receptive field (siphon and mantle shelf) of the gill withdrawal reflex

The resulting EPSP gradually decreased in size and the number and frequency of the evoked spikes decreased when the tactile stimulus was repeated. Restoration of reflex responsiveness produced either by rest or by a dishabituatory stimulus was associated with an increase of the evoked EPSP and an increase in the number and frequency of spikes in the gill motor neurons.

Since the evoked gill contraction was determined by the output of several motor neurons, there was not a perfect correlation between the spiking in any one motor neuron and the magnitude of the gill contraction. However, the correlation was reasonably close, particularly for the initial second of the spike discharge that followed a tactile stimulus. A decrease of one or two spikes within the initial discharge was usually associated with a measurable decrease of the gill contraction. The sensitivity of gill contraction to small changes in firing rate or to total number of spikes in gill motor neurons could be demonstrated independently by means of intracellular stimulation of single gill motor neurons. Variation in the amount of gill contraction was caused by variations of a few spikes in a 1-second train of directly evoked spikes in cell L7. In some circumstances, an individual spike in cell L7 produced a visible twitch of the gill. These results indicated that relatively small changes in EPSP amplitude at gill motor neurons will lead to a change in the magnitude of the gill contraction.

Habituation is a direct result of a decrease of the excitatory synaptic potentials at gill motor neurons, whereas dishabituation is due to an increase of the excitatory synaptic potentials.

Sensitization

Sensitization is a somewhat more complex form of nonassociative learning in which an animal learns to *strengthen* its defensive reflexes and to respond vigorously to a variety of previously weak or neutral stimuli, after it has been exposed to a potentially threatening or noxious stimulus. Thus, the siphon-and-gill withdrawal reflexes are enhanced if a noxious stimulus is presented to the neck or tail. Depending on the number and intensity of the sensitizing stimuli, this enhancement, like habituation, persists from minutes to weeks (Hawkins & Kandel, 1984.)

The short-term form of sensitization (minutes to hours) involves the same cellular locus as habituation, the synapses that the sensory neurons make on their central target cells, and again the learning process involves an alteration in transmitter release—in this case, an enhancement in the amount released. Sensitization also uses more complex molecular machinery. This machinery has at least five steps: (a) stimulating the tail activates a group of facilitator neurons that synapse on or near the terminals of the sensory neurons and act there to enhance transmitter release. This process is called presynaptic facilitation. (b) The transmitter released by the facilitator neurons, which is presumed to be serotonin or a related amine, activates an adenylate cyclase that increases the level of free cyclic adenosine monophosphate-activated protein kinase (AMP) in the terminals of the sensory neurons. (c) Elevation of free cyclic AMP in turn, activates a second enzyme, a cyclic adenosine monophosphate (cAMP)-dependent protein kinase. (d) The kinase acts by means of protein phospohorylation to close a particular type of K^+ channel and thereby decreases the total number of K^+ channels that are open during the action potential. (e) A decrease in K^+ current leads to broadening of subsequent action potentials, which allows a greater amount of Ca^{++} to flow into the terminal and thus enhances transmitter release.

Long-term sensitization appears to involve changes at the same locus, because it is accompanied by an increase in the number and size of active zones at sensory neuron synapses speculate that an increase in cAMP levels may trigger these long-term changes in the sensory neurons in parallel with the short-term changes.

Considerable progress has been made in explaining the neural basis of habituation. One explanation researchers have developed holds that one component of the siphon duration and gill amplitude provides a reliable index of the other.

Following the word of Pinsker et al. (1970) and Carew et al. (1979), Stopfer and Carew (1987), assessed this hypothesis on restrained animals; the tails were pinned to the wax bottom of the aquarium and the parapodia and mantle were retracted to reveal the gill. This preparation was used to simultaneously measure the amplitude of gill withdrawal and the duration of siphon withdrawal.

Once restrained, two different experimental procedures were used (a) input-output experiments consisted of an ascending series of stimuli, each stimuli was separated by a 7-minute rest period. A weak electrical stimulus was delivered to the siphon via a silver wire electrode to elicit withdrawal reflexes; (b) habituation experiments consisted of a series of 10 tactile stimuli to the siphon at a 30-second interstimulus interval (ISI). For these experiments, the siphon was brushed with a soft nylon bristle to evoke reflex withdrawals; a nylon bristle was used so that the results could be directly compared with previous work examining learning in this reflex.

The correlation between gill amplitude and siphon duration, across a broad range of response magnitudes of the withdrawal reflex was found by Stopfer and Carew, (1987). Specifically, in all experiments, the correlations were statistically significant in six out of seven preparation; the response measures of gill amplitude and siphon duration were highly correlated. The average correlation across all experiments indicated that there was a clear positive covariation between the two response measures. In the second series of experiments, habituation of the reflex response was produced by a weak, constant intensity stimulus. In all five experiments, significant habituation was evident by using either the measure of gill amplitude or siphon duration, t(4) = 19.0 and 11.0, respectively, $p < .005$ in each case. Thus, during a simple form of learning such as habituation, either of the two response measures appears to provide a highly reliable index of the reflex responses.

To further assess, Eberly and Pinsker (1984) examined the Interneuron II (Int II), a central pattern generator (CPG) that is located in the abdominal ganglion, which spontaneously generates large stereotyped contractions of the gill and siphon. The characteristic bursts of synaptic excitation and inhibition in individual siphon and gill motor neurons is associated with spontaneous contractions. Thus, Eberly and Pinsker (1970) believed that the amplitude of gill withdrawal and the duration of siphon withdrawal in response to different stimulus intensities depended on whether an Int II burst was triggered.

Intact *Aplysia* implanted with cuff electrodes on the siphon nerve were analyzed. Specifically, centrally mediated responses of both gill and siphon were examined. Thus, siphon stimuli were jets of seawater delivered by a hand-held tube connected to a pressure chamber with a valve. Spontaneous and elicited gill contractions recorded on videotape were measured from single frames on a video monitor by tracing the outline of the gill on onion skin paper and comparing the weights of cutouts from frames showing relaxed and contracted gills. The time from stimulus onset to the reappearance of the siphon between the parapodia was a measure of siphon withdrawal. Additionally, entrainment and habituation training involved different stimulus intensities, duration, and interstimulus intervals (ISIs) on a total of 58 animals throughout six, 10-trial experiments.

Eberly and Pinsker (1984) found that habituation was represented in the reflex resulting from mono-and early polysynaptic components. The contribution of the Int II component to the reflex response to a stimulus series demonstrated that some animals showed no Int II bursts on any of the trials. When presented with a series of very weak stimuli, Int II bursts were not triggered in 3 of 8 animals. On at least one trial, the remaining 5 animals showed triggered Int II bursts. For these animals, the dropping out of the triggered Int II component is correlated with the initial rapid decrease in response duration ($r_s = .97$, $p < .005$). Response durations with triggered bursts are significantly longer than those without ($p < .01$). These data demonstrate the additional influence of the Int II component on reflex dynamics. Thus, when Int II bursts are initially present, reflex habituation is much more dramatic than that which can be attributed to other reflex components.

As evidenced by the work of Kandel and countless others, the behavioral plasticity of invertebrate behavior has been well established in *Aplysia*. Thus, researchers have drawn comparisons to other species, to suggest that similar phenomena occur. Specifically, researchers have used different species of annelids to study characteristics of habituation, retention of habituation, and a number of variables that affect these processes.

Hirudo medicinalis

The leech, which belongs to the annelids phylum, has been found to habituate to a number of different stimuli. Additionally, when localized to the leech body wall, the stimuli can evoke a variety of behavioral responses, including bending (contraction from stimuli), shortening (abrupt longitudinal contraction), and swimming (the elongation, flattening, and undulation of leech body); each of these responses may occur alone or in combination with others as part of a complex behavioral sequence (Gee, 1913).

Research in leech neurobiology began with the pioneering work of W. Gee who first demonstrated that progressively lesser disruptions of an ongoing ventilator response were the result of repetitive presentations of a shadow or a jarring motion. Kaiser extended these findings and showed that the rate of habituation to the shadow was negatively correlated to the intensity of light. Similarly, Lockery et al. (1985) demonstrated habituation of the photic-elicited shortening response and Stoller and Sahley (1985) described habituation, sensitization, and classical conditioning of the touch-elicited shortening response in the intact animal. In addition to modulating the shortening reflex, habituation has been observed to affect more complex behaviors, such as the shock-elicited swimming response (Debski and Friesen, 1985) and the motor response elicited by water current (Ratner, 1972.)

Although a number of different leech species exist, species of the order *Hirudinea* are considered to be the most advanced annelid, as well as the most common; more specifically, *Hirudo medicinalis*, or the European medicinal leech, is a common variety of the Hirudinea order.

In general, the nervous system of the leech, a segmented worm, is organized into 32 body segments; of which 4 form the head, 7 fused ganglia (mass of connected nerve cells) form the tail, and the remaining 21 form iterated midbody segments (Figure 3). These ganglia form a chain which lies along the ventral part of the leech. Two large lateral bundles of nerve fibers and a thin medial connective fiber compose the connectives, which join the ganglia. Referred to as the ventral blood sinus, the entire nerve cord is enclosed in a network of blood vessels. A pair of nerve roots arise and branch to segmental structures in that region. Additionally, each segmental ganglion contains about 400 nerve cell bodies, with slight variation from ganglion to ganglion.

Influenced by the work of Gee, Nicholls & Baylor (1968) indicated that the first neurons to be identified in terms of function were the sensory neurons that respond to touch, pressure, and to mechanical stimulation of the skin. These are the T, P, and N cells respectively. Moreover, Nicholls & Purves (1970) demonstrated that each of these sensory cells was monosynaptically connected to the L cell, a motor neuron which subserves the shortening reflex. In addition, Kristan (1982) revealed that co-activation of T and P cells was necessary and sufficient to produce bending. Finally, intracellular stimulation of any one of these three sensory neurons in a semi-intact preparation can evoke swimming activity (Debski & Friesen, 1982).

Accordingly, it is important to note that, in most instances, recording neural activity involves opening up the animal. This presents a complicated and in-depth process, which exposes part of the nervous system. Using semi-intact animal preparations allows researchers to characterize movements of the animal over time, to determine the probable sequence of muscle activation during particular behaviors, to measure the motor neuronal firing patterns as the intact part of the animal performs the behaviors, to characterize the neuronal interconnections underlying behaviors in an isolated nerve cord, and to confirm observations (Esch & Kristan, Jr., 2002). Furthermore, researchers have found motor neuron firing patterns that would produce swimming (Kristan et al., 1974), shortening (Shaw & Kristan, 1995, 1999), and crawling (Baader, 1997) if that part of the animal were still intact. Similarly, researchers have found that the same firing patterns were seen in the completely isolated nervous system (Kristan & Calabrese, 1976; Eisenhardt et al., 2000).

Similar to the gill-and-siphon withdrawal reflex of *Aplysia*, the shortening reflex of the leech has been the main focus of many researchers, who have suggested that the behavioral correlates of such phenomena may actually be habituation and sensitization of the shortening reflex (Magni & Pellegrino, 1975). Thus, demonstration of nonassociative modification of this reflex could serve to link the cellular studies already in progress with behavioral events. Since much of the neural circuitry of the shortening reflex is already known, the result would be a well-charted reflex circuit in which to study the neural basis of nonassociative learning (Kramer, 1981; Kristan, 1982.)

Ratner (1972) focused his attention on the responses of movements to repeated light onset with the leech species, *Macrobdella decora* (Ratner, 1972); the same leech species that is described by Mann (1962). More specifically, Ratner investigated habituation and retention of habituation of responses to these stimuli.

Prior to the experiment, Ratner created controlled conditions similar to ones described by Lockery et al. (1985). For instance, Ratner created temperature-controlled chambers in order to overcome the difficulty in reliably eliciting contractions to stimuli; the temperature of each chamber ranged from 20° –21° C. Additionally, different levels of light onset were used to produce consistent conditions: once placed in temperature-controlled chambers, the specimens were placed on the floor and were covered at all times by a sheet of opaque black plastic; when a beaker containing a specimen was moved, illumination from a ruby-red bulb was used. Moreover, Ratner described the criterion for a response, habituation, and retention of habituation: the topography of a response to light typically consisted of the worm's releasing the anterior sucker form the side of the beaker and attaching it in a different location; the criterion for habituation was eight consecutive trials with no movement response after which the beaker containing the worm was returned to the floor under the black plastic sheet, and another worm in its beaker was taken for testing for original habituation; tests for retention of habituation were conducted exactly like tests for original habituation (Ratner, 1972). Finally, two retention intervals were established for each worm: the first retention test was conducted 24 hours after original habituation, and the second retention test was conducted 48 hours after original habituation.

In general, the results from Ratner's first experiment demonstrated habituation and retention of habituation of responses to light onset. More specifically, the results from the habituation phase of the experiment showed that all worms respond initially to an increase in the level of illumination, and that the frequency of responses increased temporarily from the first to the second block of trials before gradually decreasing to zero for all worms (Ratner, 1972). Furthermore, the fact that retention of habituation was found over two successive 24-hr. period extends the generality of evidence for retention of habituation by annelids, and provided the first evidence of retention of habituation with a species of leech. The results also provided clear evidence of retention of habituation of responses that were elicited by an increase in illumination for a species of annelid. Finally, the results bear on the generality of Ratner's analysis of retention of habituation in which retention is assumed to be determined by the strength of the eliciting stimulus, where the stronger the eliciting stimulus, the less the retention of habituation. According to this view, habituation of responses to light can be retained for 24 hours if the light is a moderate or weak elicitor of responses, as in the present study.

In his second experiment, Ratner studied leech responses to pulses of water current and habituation of responses to these stimuli. Additionally, as a further step in the investigation of the characteristics of habituation with repeated stimulation, Ratner tested two groups of leeches with different intertrial intervals; intertrial intervals (ITI) appeared to be a relatively robust variable in studies of habituation including those using annelids (Ratner, 1970).

Overall, the results provide evidence that leeches respond to changes in water currents and that the responses were similar in topography to the responses of *Dina microstoma* as described by Gee (1912). That is, on trials early in habituation the worms responded by swimming against the water current.

Lockery, Rawlins, and Gray (1985), who agreed with this demonstration, studied the shortening reflex of the *Hirudo medicinalis* in response to photic stimuli. Lockery et al. (1985) studied four types of learning in three separate experiments: short-term habitation, long-term habituation, dishabituation, and sensitization.

In their first experiment, Lockery et al. (1985) distinguished between short and long term habituation; short-term habituation was observed at interstimulus intervals (ISI) of the order of seconds or minutes, while long-term habituation was observed at interstimulus intervals of the order of hours or days (Kandel, 1976.) Moreover, it was noted that activity levels could be reduced by storing the animals at low temperatures (5-7 °C); this plan was necessary in reliably eliciting contractions to photic stimuli since active animals were less likely to contract than active ones. Thus, animals were studied either in stored containers at room temperature or in cold conditions.

The study indicated the presence of short-term habituation by the decline in the number of shortening responses occurring in successive 10-trial blocks. Statistically, short-term habituation was demonstrated by the significant effect of blocks, $F(3, 324) = 47.15, p < .001$. Storage temperature clearly affected short-term habituation as it was also statistically significant, $F(3, 324) = 3.97, p < .01$. On the other hand, long-term habituation did not occur as there was a steady linear increase in response probability over the first 6 days (Lockery et al., 1985.)

In the second experiment, Lockery et al. (1985) tested dishabituation at the behavioral level focusing on the shortening reflex. Furthermore, electric shock was chosen as the dishabituating stimulus as it appeared that the shortening reflex was mediated by a fast conducting system; electric shock created a transient longitudinal contraction (Framer, 1981). Moreover, demonstration of dishabituation at the behavioral level paralleled a previous neural analogue of dishabituation (Belardetti, Biondi, Colombaioni, Brunelli, & Trevisani, 1982), which suggested that habituation and dishabituation of the shortening reflex to certain responses were similar to habituation and dishabituation of the gill-withdrawal reflex in *Aplysia* (Kandel & Schwartz, 1982).

Although Lockery et al., found multiple results—a parametric analysis of variance revealed no effect of the dishabituating stimulus, while a Friedman analysis of the three trials before and after shock delivery demonstrated a significant Dishabituation X 3-Trial Blocks interaction (Lockery et al., 1985)—an analysis of response latencies presumes that dishabituation had occurred: immediately following shock, response latency decreased.

Similarly, the third experiment demonstrated that sensitization occurs in the shortening reflex in the medicinal leech. However, results in response frequency and response latency of the third experiment suggest that the dishabituation seen in experiment two is actually another instance of sensitization.

Overall, Lockery et al. observed short-term habituation, which repeated without significant change over successive sessions. Additionally, Lockery et al. classified dishabituation-like phenomena as sensitization and found no evidence for long-term habituation; instead, a steady increase in response frequency occurred over the course of experiment 1 and experiment 2. Furthermore, the remaining observations were accounted for based on the two processes that are well established in other species, including the *Aplysia*. These processes include: short-term decremental and short-term incremental, which are independent of each other and are responsible for habituation and sensitization, respectively. The simplicity of these short-term processes in the leech makes preparation particularly suitable for electrophysiological investigations into the neural basis of habituation and sensitization. (Lockery et al., 1985).

In most investigations of leech habituation, researchers have focused on intact leeches to study the shortening reflex and subsequent forms of nonassociative learning. However, Boulis and Sahley (1988) took a different approach: a demonstration that the shortening reflex of the semi-intact animal (figure 4) also displays nonassociative forms of learning (habituation, dishabituation, and sensitization). In order for the researchers to simultaneously record behavior and cellular activity, they devised an intact preparation of the leech. First, a small incision was made at the animal's fifth annulus on its ventral side. The nerve cord (connective) was severed between the fused ganglia and the first segmental ganglion in order both to eliminate the complicating effects that might be contributed by the fused head ganglia (Kristan et al., 1982) and to incapacitate the anterior sucker.

In order to measure the shortening response, a 20cm silk suture thread was attached to the anterior sucker and to a force transducer. Last, the leech was transected at the ninth segment, leaving 2 ganglia and the associated sinus exposed. The sinus on the remaining connective caudal to the second ganglion was removed. This preparation was then secured to a Sylgard lined Petri dish with 4 stainless steel pins placed at the line of transection. The exposed ganglia were pinned out behind the animal with shortened minuten pins. The nerve connective was sucked into a suction electrode. It was necessary to leave 2 ganglia exposed in order to relieve the stress on the electrode connection. Further, the ganglion stability that is achieved in this fashion is essential to future efforts aimed at intracellular recording. The behavioral response was recorded on a Gould 2-

channel recorder. (Boulis & Sahley, 1988).

In their study, Boulis and Sahley conducted three experiments in which they tested for habituation and dishabituation in the semi-intact leech, habituation and dishabituation over various interstimulus intervals (ISIs), and sensitization of the shortening reflex. Additionally, the stimulus used to elicit shortening was a shock provided by silver wires attached to a Grass stimulator (Boulis & Sahley, 1988).

The results from the three experiments clearly indicated that the touch-elicited shortening response of the semi-intact leech preparation was modified by habituation, dishabituation, and sensitization (Boulis & Sahley, 1988). Additionally, the observed plasticity of the semi-intact preparation was quite similar to that observed in the intact animal (Stoller & Sahley, 1985). Habituation of the response occurred following 20 presentations of a tactile stimulus applied to the anterior dorsal surface, and dishabituation occurred following the presentation of a noxious stimulus. Moreover, the degree of habituation was dependent on the intertrial interval (ITI). That is, habituation training resulted in progressively more habituation as the ITI went from 10 seconds to 5 minutes. In fact, no behavioral decrement occurred with a 10 second ITI (Boulis & Sahley, 1988). Furthermore, the reported data suggested that two independent processes, habituation and sensitization, interacted in the final determination of a behavioral response; Boulis and Sahley suggested that the characteristics of the stimulus, the frequency and intensity, were critical determinants of the observed behavior. Finally, the individual results from the third experiment indicated that presentation of a noxious stimulus prior to habituation training prevents subsequent habituation, in contrast to the case of leeches in a control group not experiencing the noxious stimulus.

In subsequent investigations of the semi-intact leech preparation, several researchers have focused on one type of behavior: swimming evoked by low-intensity tactile stimulation. Studies have shown that the swimming response subsequently recovers spontaneously or can be restored quickly by administration of a strong stimulus. Thus, the decrement in the behavioral response (swimming) conforms to the operational definition of habituation.

To swim, a leech flattens its tubular body and undulates dorsoventrally; wave crests and troughs form alternately at the anterior end and pass backward along the animal, thus producing the forward thrust. These crests and troughs are formed by localized shortenings of the dorsal and ventral body wall. These same movements still occur after the head and tail brains have been disconnected from the ventral cord. Movements in each of the 21 segments are effected by muscles largely restricted to that segment and each set of muscles is controlled by motor neurons within its own segmental ganglion.

In particular, three distinct sets of muscles are used to produce swimming movements (Figure 3.) One set runs transversely across the body from the dorsal to the ventral body wall. These dorsoventral muscles contract throughout the swim in all segments, causing flattening as well as elongation of the body. The other two sets lie longitudinally in the body wall, so that their contraction causes a shortening of the segment. One set of these longitudinal muscles lies ventral to the lateral edge; its contraction in one segment produces a body crest. The other set lies dorsal to the midline; its contraction produces a body trough. During swimming, a single segment produces alternate contractions of the dorsal and ventral longitudinal muscles. The rearward travel of crests and troughs results from an antero-posterior progression of this contraction cycle in successive segments (Kristan, Jr., 1974).

In investigations of the semi-intact leech focusing on a swimming behavioral response, researchers are actually focusing on an ensemble of rhythmically active motor neurons, which generate the contractile rhythm of the dorsal and ventral longitudinal muscles. This motor neuron ensemble consists of dorsal and ventral excitors and dorsal or ventral inhibitors. The excitors cause contraction of the longitudinal muscles in dorsal or ventral territories of the body wall via direct excitatory synaptic input. The inhibitors cause relaxation of the longitudinal muscles in corresponding body wall territories by inhibitory synaptic inputs both to the muscle fibers at the periphery and to the homonymous excitors within the segmental ganglion. Thus during the troughs or dorsal phase of the segmental swim cycle, an impulse burst of each of four dorsal excitors contracts the dorsal body wall longitudinally while an impulse burst in at least one ventral inhibitor relaxes the ventral body wall and inhibits the activity of the ventral excitors. On the other hand, during the crest, or ventral phase of the cycle, an impulse burst in each of the three ventral excitors contracts the ventral body wall longitudinally, while an impulse burst in each of two dorsal inhibitors relaxes the body wall and inhibits the activity of the dorsal excitors (Kristan, Jr., & Calabrese, 1976).

Furthermore, Debski and Friesen (1985) conducted an experiment in which the body wall flap of the leech was attached to an otherwise isolated ventral nerve cord. Therefore, the stimulus was a light stroke applied repeatedly to this body wall flap. In preparing the leech, Debski and Friesen (1985) used a method similar to that of Boulis and Sahley (1988): the nerve cord or connective was severed between the head and the first segmented ganglion, and the body wall flap was attached to ganglion 11; this flap was at least three segments long, was centered about segment 11, and extended from the dorsal to the ventral midlines. Results showed that at first, a light stroke to a body wall flap was an effective stimulus for eliciting swimming activity in a nerve cord-body wall preparation. However, with repeated stimulation, swim lengths declined and then, quite abruptly, stroking the body wall no longer evoked a swimming response.

In another investigation, Kristan, Jr. (1974) took an in-depth approach regarding the leech muscles and motor neurons responsible for swimming behavior. Additionally, he suggested that a reciprocal stretch reflex involved those same muscles and motor neurons. The reflex played an important role in generating the swim cycle.

Following the work of Gray et al. (1938), Kristan severed anterior and posterior brains from the ventral nerve cord to record the activity of the nervous system during swimming. The body wall of five midbody segments were cut away, leaving the ventral nerve cord with lengths of peripheral nerves intact; sometimes, one side of the body wall of one segment was left attached to its ganglion by the segmental nerves. Tactile stimulation of the posterior segments induces the intact parts to carry out swimming movements in their normal coordinated sequence. A light was shone across the front segments onto a photocell to produce an electrical record of swimming movements in the intact parts of the body. Furthermore, the dissection of the middle segments allowed three types of recordings to be made: tension generated by muscles in the body-wall flap, intracellular electrical activity from nerve cell bodies or muscles fibers, and extracellular electrical activity from segmental nerves.

On the basis of such recordings, Kristan, Jr. identified 10 cells as motor neurons generating the rhythmic swimming movements: five produce dorsal spike bursts, four are dorsal muscle excitors and one is a ventral muscle inhibitor; four produce ventral spike bursts, three are ventral muscle excitors, and two are dorsal muscle inhibitors. Additionally, two kinds of connections were found: excitors of the same set of muscles were linked by electronic junctions, and each inhibitor was connected via a central inhibitory path to the excitors of the same set of muscles which the inhibitor inhibits peripherally (Kristan, Jr., 1974). Overall, Kristan's findings indicate that the bursts in the inhibitors make the muscle contractions rhythmic, allowing the leech to swim.

In the present study I will attempt to investigate these issues further by (a) attempting to measure habituation, through repeated electrical stimulation presented at different intensities across different locations on the leech and (b) replicating Kristan, McGirr, and Simpson's (1982) study to determine if electrical stimulation to the posterior region of the leech produces a swimming response.

Based on the past studies, I predict that when presented with electrical stimulation to the posterior region of the leech, a swimming behavior will occur. Additionally, I predict that habituation of the swimming response will occur after repeated stimulation; as characterized by a gradual decrease in swimming duration and length, or distance covered. Thus, results will reveal:

1. Consistent with past research, *Hirudo medicinalis* demonstrate nonassociative forms of learning, specifically habituation and sensitization (Kristan et al., 1982).

2. *Hirudo medicinalis* exhibit a pattern of responses to the stimulus trains of different intensity at different locations.

3. Behavioral responses and learning behaviors in *Hirudo medicinalis* parallel similar phenomena in *Aplysia californica*.

II. Materials and Methods

Subjects. Leeches, *Hirudo medicinalis*, were obtained from a commercial supplier and maintained at room temperature (20°C) in cloth-covered glass aquaria, filled with approximately 5 cm of non-chlorinated tap water. The leeches were unfed. All experiments were performed on intact leeches.

Apparatus. At the start of the experiment, all animals were maintained in a glass aquarium filled with 5 cm of non-chlorinated tap water; the glass aquarium was fitted with lined measurements to determine swim length. Leeches were allowed to acclimate for approximately one day after arrival. The habituating stimulus was an electrical stimulation delivered through tapered electrodes with their tips approximately 1mm apart. A Grass SD $_9$ stimulator was used to produce 1ms electric pulses, at 10 Hz. To be sure that the observed behavioral responses were to electrical and not to mechanical stimulation, the electrodes were placed gently on the skin with the stimulator turned off. There was usually no response to the electrode placement, and the electrical stimulus was delivered about 3s after the electrode made contact. If there was a response to touch, the electrical stimulus was delivered about 60 s after completion of the touch-elicited response.

Procedure: After completion of the acclimation period, stimuli were presented at 0.5V gradations, ranging from intensities which did not elicit a response, usually about 3.5V to intensities above which no new behavior could be produced, which was no higher than 12V. After each 1 ms presentation, leeches were observed for 1 min before the next presentation. Similarly, 3 m elapsed between trials; the three trials dictated the area of the leech that was stimulated: trial 1-posterior, trial 2-middle, and trial 3-anterior. Overall, each leech received 5 different intensity presentations, three times. After completion of the three-trial process, leeches were moved to a glass cylinder filled with 500 mm of non-chlorinated tap water.

Dependent measure: the dependent measure was the amount of swimming elicited by the conditioned stimulus recorded as the length and duration of the behavior, as observed by the researcher and video camera.

III. Results

Nomenclature. The methods I used to classify measures and variables followed those used by Kristan et al. (1982) with multiple levels of intensity (5) and multiple levels of trials (3), which also corresponded to the location of stimulus presentation. Additionally, dependent variables consisted of duration and length, or distance covered.

Statistical analysis. After conducting a within-subjects, repeated measures analysis of variance (ANOVA), results showed a significant effect between intensity and length, or distance covered, $F(1,3) = 201.01$, $p = .001$. Overall, significant mean differences demonstrated that leeches covered the greatest distance at higher intensities. A graph of the average mean lengths for all trials and locations of stimulation can be seen in Figure 6.

Results for acquisition of habituation. After statistical analysis, the results did not indicate a strong effect between length covered and trial number. Instead, it suggested that leeches possess the potential for habituation, since the means for intensities 6.6 v and 7.5 v suggest patterns of habituation (Figure 7.) Overall, habituation was not elicited.

Results for swimming behavior. Further comparisons showed that swimming length covered was the greatest for Trial 1, which corresponded to the area in which the stimulation was presented; in this instance, posterior (Figure 5.) Similar results showed that overall, the longest length covered for swimming occurred with stimulation to the posterior area of the leech (figure 7) versus the middle or anterior regions.

IV. Discussion

As hypothesized, I found that electrical stimulation presented to the posterior region of the leech produces a swimming response. This finding is consistent with the existing literature (Kristan et al., 1982), and serves to further affirm the general principal that *Hirudo medicinalis* show a pattern of responses to the stimulus trains of different intensity at different locations. For instance, on all of the trials where electrical stimulation was presented to the posterior region, the mean lengths were the highest: intensity 3.6 ($M = 49.59$), intensity 4.6 ($M = 54.59$), intensity 5.6 ($M = 47.34$), intensity 6.6 ($M = 57.09$) and intensity 7.5 ($M = 67.92$) (Figure 7.) This finding is not surprising as Kristan et al. (1982) demonstrated that stimuli between 4.5 and 7 V delivered to the posterior end elicited swimming or in some instances, curling followed by swimming. Similarly, it was suggested that behavioral differences were due to connections of the mechanosensory neurons in different body regions on to different interneurons.

I also found that as the stimulus intensity increased, there was a progression in the response type. For instance, when applied to the anterior end, the lowest stimulus intensity of 3.6 v elicited a swimming response with less vigor and intensity as compared to a high intensity of 7.5 v. In many instances, vigorous swimming was observed, which consisted of rapid swimming.

Although a swimming response was elicited, habituation was not. This was not all that surprising because numerous studies (Kristan et al., 1982; Debski & Friesen, 1985; Ratner, 1972) elicited habituation in the semi-intact leech preparation. For instance, Debski and Friesen (1985) demonstrated that tactile stimulation of a body wall flap attached to the ventral nerve cord of the medicinal leech evoked episodes of swimming activity. This swimming response experienced habituation, involving changes in swim initiation and swim maintenance.

One possible explanation for this finding can be drawn from the fact that some leeches were more active then others. Although the leeches were tested at the beginning to ensure that the observed response was behavioral and not mechanical, there was no way to ensure that all leeches, on each trial, did not respond to mechanical stimulation; in instances that this did occur, leeches were given a 1-minute rest period with the electrodes on the leech and then stimulated.

Another possible explanation lies in the fact that little information is known about leech social interactions and patterns of communication. If leeches do possess some method of communication, this would clearly alter my finding. However, when trials were completed for each leech, they were moved to a separate location to prevent repeated stimulation; the observed behaviors and responses did not vary for leeches stimulated at the beginning (with other leeches) or leeches at the end (without other leeches).

Throughout the study, most of the responses I observed were expected. However, an interesting finding that I can across involved classical conditioning or sensitization patterns within leeches. With the repeated presentation of different intensities over a period of time, the swimming behavior did not elicit habituation, but the leeches did learn to recognize that the electrodes produced a negative consequence. Thus, at higher intensities when stimuli have already been presented multiple times, the leech contracts at the slightest touch of the electrodes, without deliverance of a shock. These observations parallel many behaviors seen in *Aplysia*.

Specifically, Hawkins, Greene, and Kandel (1998) studied classical conditioning, as well as differential conditioning and second-order conditioning of the gill-withdrawal reflex. They examined the gill withdrawal reflex in a dissected preparation of *Aplysia*, when it was stimulated with a conditioned tap, controlled by a force stimulator. Additionally, an electric shock delivered to the mantle shelf was the uncontrolled stimulus; during paired training, the tap began 500 ms before the shock and during unpaired training, the interstimulus interval was 2.5 min.

Their first experiment, which consisted of four groups of animals with 15 animals per group, indicated that paired training produced an increase in the response to the tap that was most reliable on the final posttest. There was a significant overall difference between the groups, F (3, 56) = 6.66, p, < .001 and paired training produced a significantly greater enhancement of gill withdrawal than each of the other training conditions. These results demonstrate classical conditioning of the gill-withdrawal reflex (Hawkins et al., 1998).

Overall, the study demonstrated that correlations exist between the types of learning that occur within *Aplysia californica* and *Hirudo medicinalis*. Similarly, the study proves that *Hirudo medicinalis* follow patterns of responses to stimuli of different intensities and presented at different locations.

Lastly, to make a truly valid argument further research should be conducted with both intact and semi-intact leech preparations to determine the types of learning that occur.

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Figure Caption

Figure 1. Anatomy of *Aplysia californica*. Dorsal view of an intact *Aplysia*. The parapodia and mantle shelf have been retracted to allow direct observation.

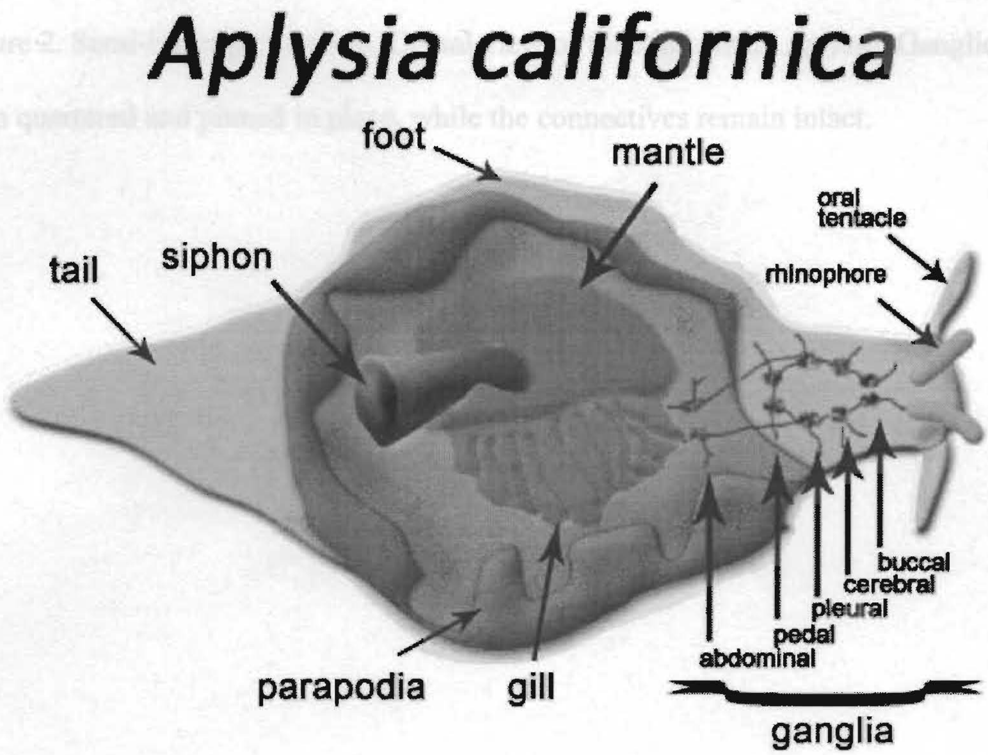

Aplysia californica
foot
mantle
oral tentacle
rhinophore
tail
siphon
buccal
cerebral
pleural
pedal
abdominal
parapodia
gill
ganglia

Figure Caption

Figure 2. Semi-intact preparation. Dorsal view of the semi-intact *Aplysia*. Ganglion has been quartered and pinned in place, while the connectives remain intact.

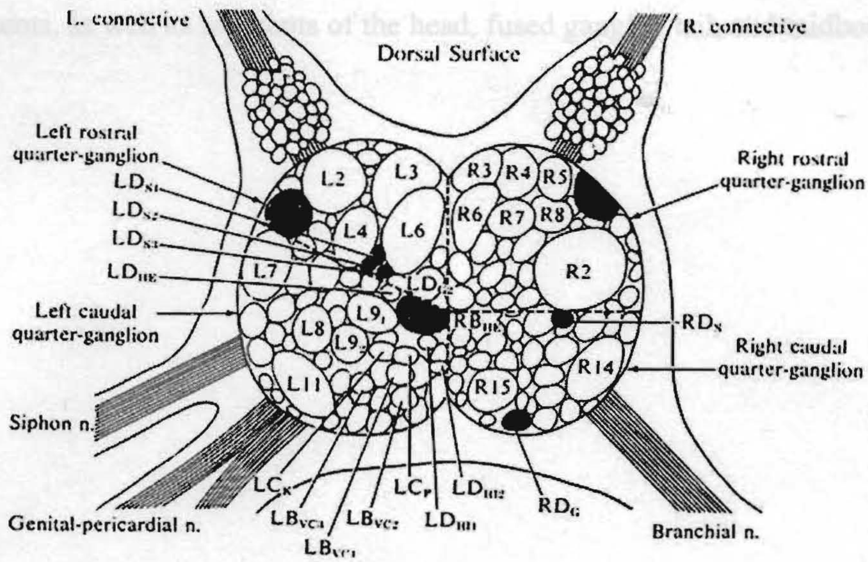
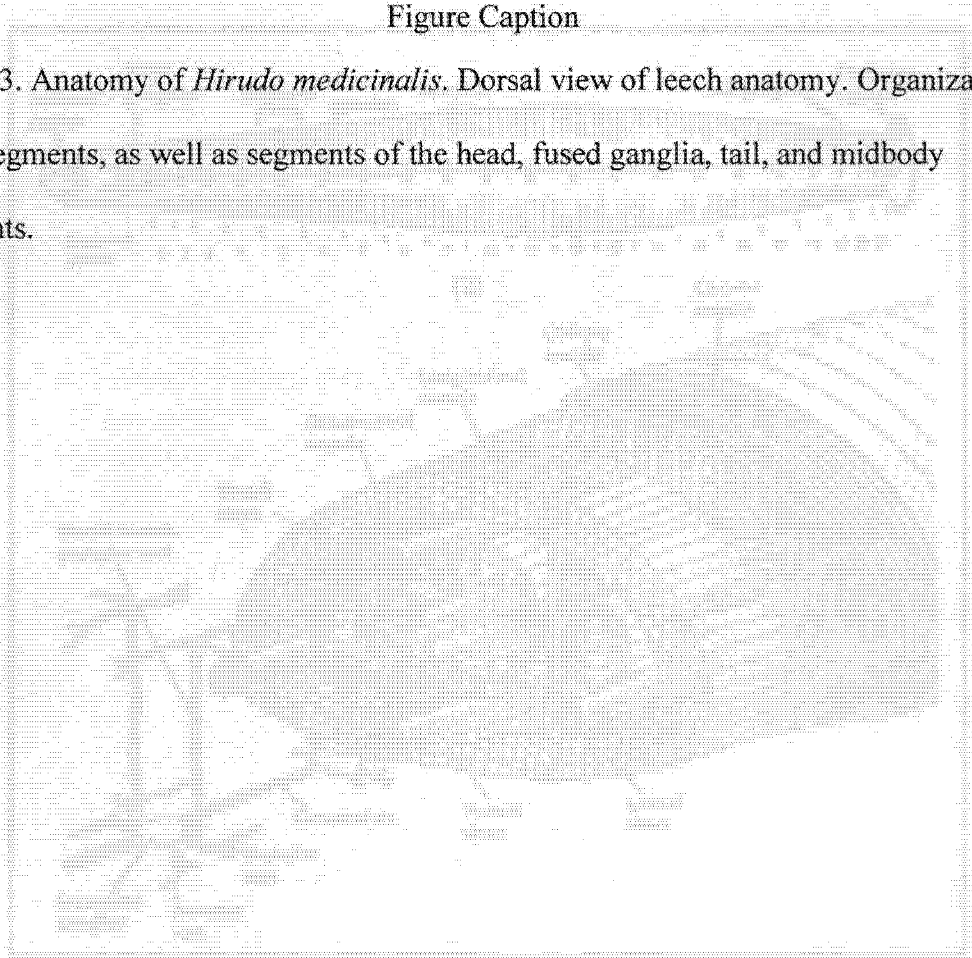

L. connective
Dorsal Surface
R. connective
Left rostral quarter-ganglion
Right rostral quarter-ganglion
L2
L3
R3
R4
R5
L4
L6
R6
R7
R8
L7
R2
L8
L11
R14
R15
Left caudal quarter-ganglion
Right caudal quarter-ganglion
Siphon n.
Genital-pericardial n.
Branchial n.

Figure Caption

Figure 3. Anatomy of *Hirudo medicinalis*. Dorsal view of leech anatomy. Organization of body segments, as well as segments of the head, fused ganglia, tail, and midbody segments.



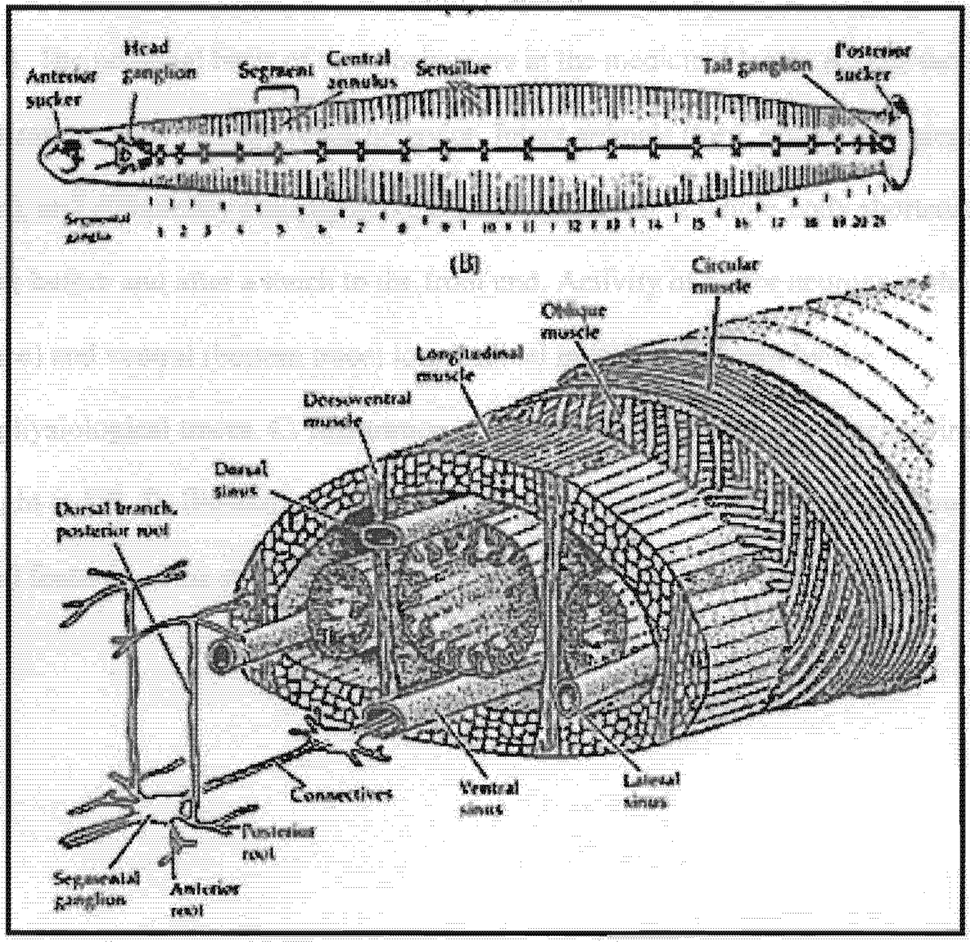

Anterior sucker
Head ganglion
Segment
Central annulus
Sensillae
Tail ganglion
Posterior sucker
(B)
Circular muscle
Oblique muscle
Longitudinal muscle
Dorsoventral muscle
Dorsal sinus
Dorsal branch, posterior root
Connectives
Ventral sinus
Lateral sinus
Posterior root
Segmental ganglion
Anterior root

Figure Caption

Figure 4. The neuronal basis of three behaviors in the medicinal leech. **A.** The three types of preparations used to study leech behavior. Intact animals (top), semi-intact preparation (middle), and completely isolated nerve cord (bottom). **B.** A whole-body shortening response before and after a touch to the front end. Activity of motor neurons to the dorsal (top trace) and ventral (bottom trace) longitudinal muscles as shown by the electrophysiological traces. **C.** Illustration of 12 successive frames of a leech swimming from right to left. **D.** Illustration of left-to-right crawl step. The six drawings show essential features of the step.

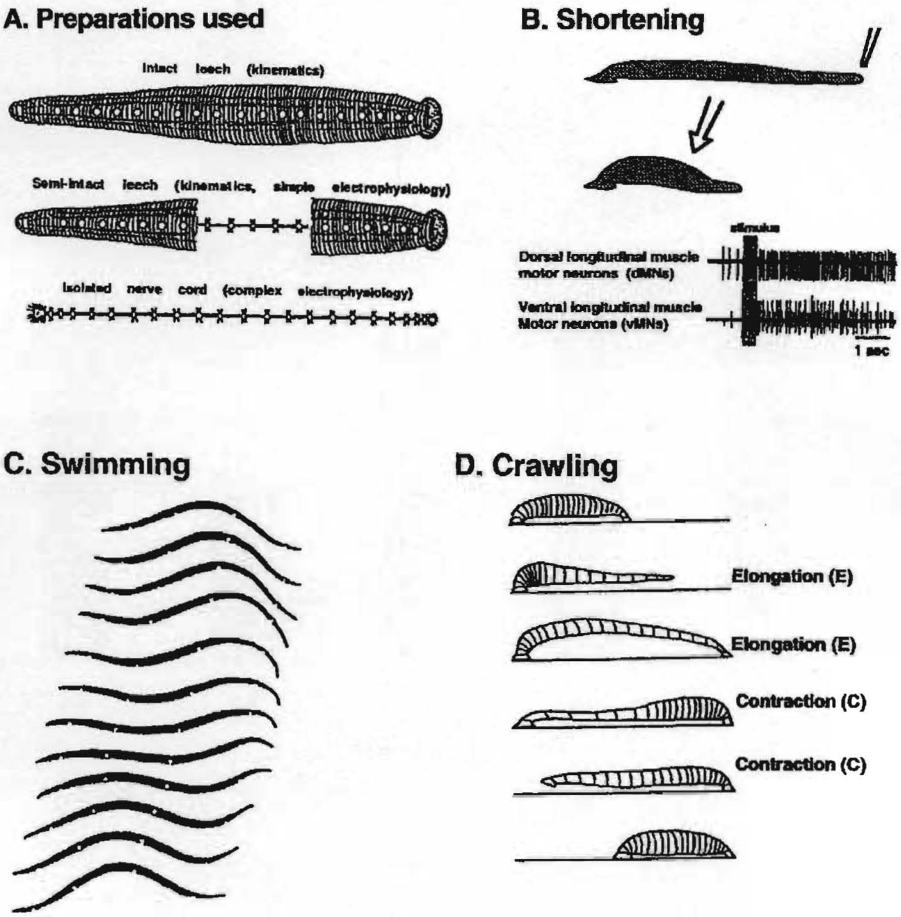

A. Preparations used
Intact leech (kinematics)
Semi-intact leech (kinematics, simple electrophysiology)
Isolated nerve cord (complex electrophysiology)
B. Shortening
stimulus
Dorsal longitudinal muscle motor neurons (dMNs)
Ventral longitudinal muscle Motor neurons (vMNs)
1 sec
C. Swimming
D. Crawling
Elongation (E)
Elongation (E)
Contraction (C)
Contraction (C)

Figure Caption

Figure 5. The average mean distance covered for all intensities and trials.

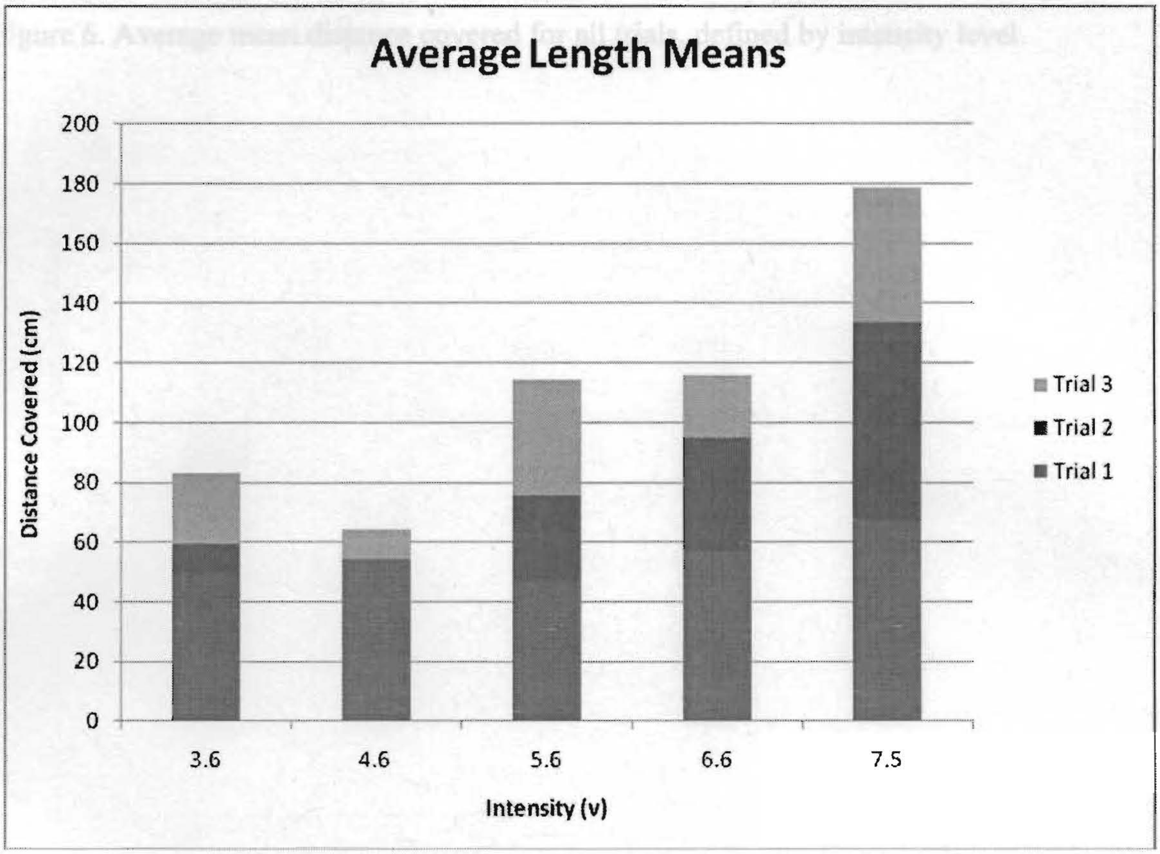

Average Length Means
Distance Covered (cm)
200
180
160
140
120
100
80
60
40
20
0
3.6
4.6
5.6
6.6
7.5
Intensity (v)
Trial 3
Trial 2
Trial 1

Figure Caption

Figure 6. Average mean distance covered for all trials, defined by intensity level.

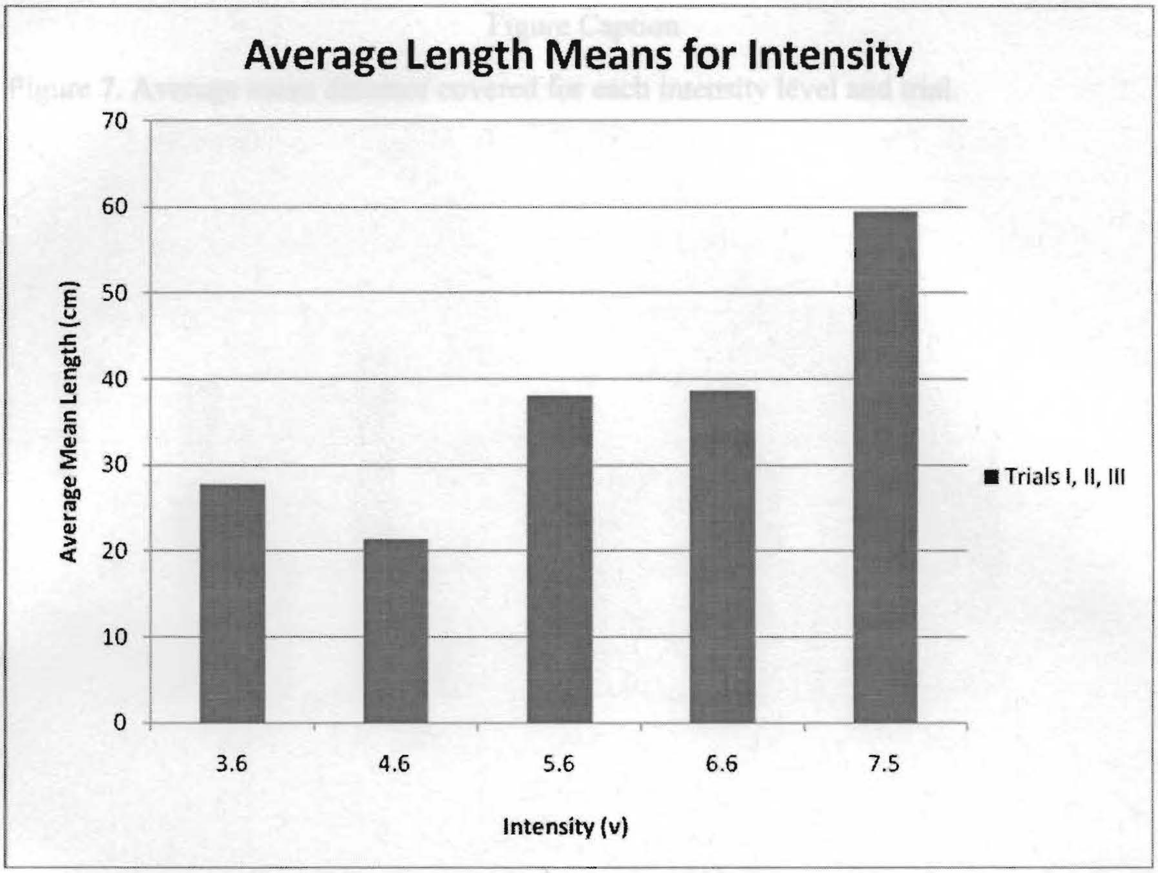
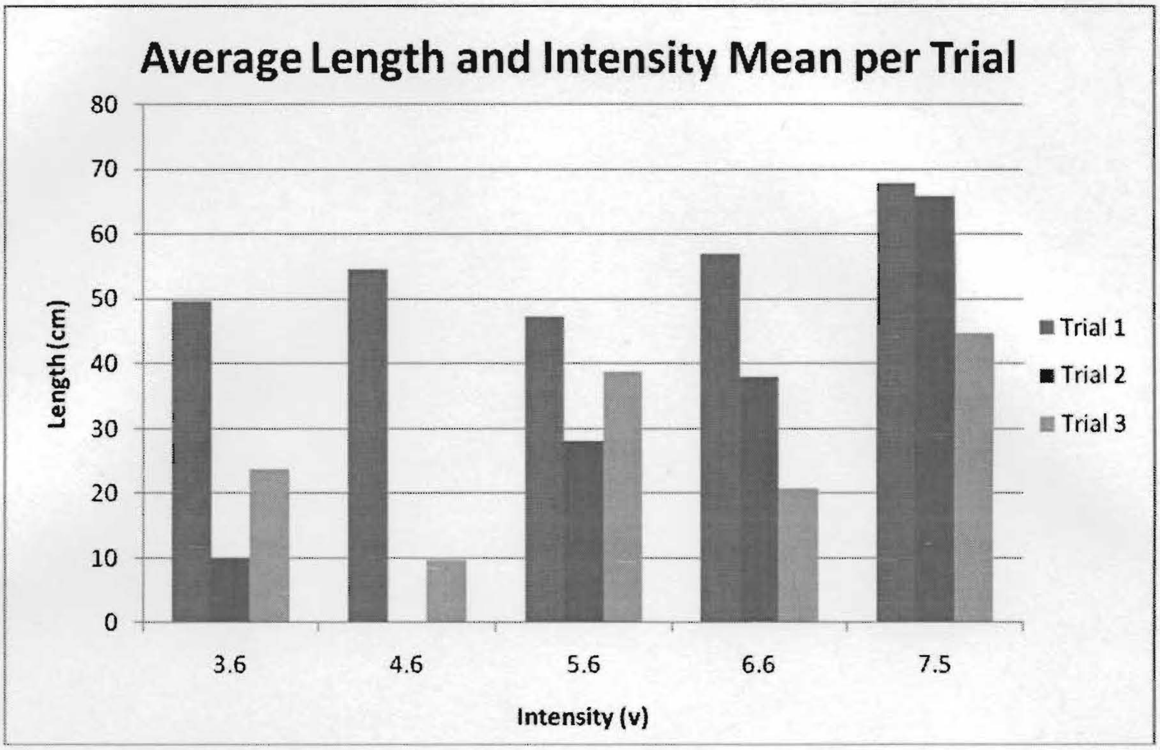

Average Length Means for Intensity
Average Mean Length (cm)
70
60
50
40
30
20
10
0
3.6
4.6
5.6
6.6
7.5
Intensity (v)
Trials I, II, III

Figure Caption

Figure 7. Average mean distance covered for each intensity level and trial.


Average Length and Intensity Mean per Trial
80
70
60
50
40
30
20
10
0
Length (cm)
3.6
4.6
5.6
6.6
7.5
Intensity (v)
Trial 1
Trial 2
Trial 3