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Physiological and developmental gradients in *Lumbriculus variegatus*

by

Nalena Lesiuk

**A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY**

Major: Neuroscience

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TABLE OF CONTENTS

CHAPTER 1. GENERAL INTRODUCTION	1
Biology of <i>Lumbriculus variegatus</i>	1
Dissertation Objectives	4
Dissertation Organization	6
References	6
CHAPTER 2. AUTOTOMY REFLEX IN A FRESHWATER OLIGOCHAETE, <i>Lumbriculus variegatus</i> (CLITELLATA: LUMBRICULIDAE)	8
Abstract	8
Introduction	9
Material and Methods	10
Results	15
Discussion	20
References	22
CHAPTER 3. BEHAVIORAL PLASTICITY AND CENTRAL REGENERATION OF LOCOMOTOR REFLEXES IN THE FRESHWATER OLIGOCHAETE, <i>Lumbriculus variegatus</i>. I: TRANSECTION STUDIES	32
Abstract	32
Introduction	33
Material and Methods	35
Results	40
Discussion	46
References	50

CHAPTER 4. BEHAVIORAL PLASTICITY AND CENTRAL REGENERATION OF LOCOMOTOR REFLEXES IN THE FRESHWATER OLIGOCHAETE, <i>Lumbriculus variegatus</i>. II: ABLATION STUDIES	60
Abstract	60
Introduction	61
Material and Methods	62
Results	66
Discussion	72
References	76
CHAPTER 5. BLACKWORMS, BLOOD VESSEL PULSATIONS, AND DRUG EFFECTS	90
Background	91
Material Required	93
Viewing Chambers	94
Stock Solutions	97
Experimental Design & Lab Groups	97
Procedures	98
Sample Results: Nicotine	101
Sample Results: Caffeine	102
Alternative Experiment	102
Discussion	103
Acknowledgment	104
References	104

CHAPTER 6. GENERAL CONCLUSION	114
ACKNOWLEDGMENTS	117

CHAPTER 1. GENERAL INTRODUCTION

Biology of *Lumbriculus variegatus*

Lumbriculus variegatus is an aquatic oligochaete in the Phylum Annelida, Order Lumbriculida. In its natural environment, *Lumbriculus* lives in submerged vegetation at the edge of freshwater ponds or marshes. A special characteristic of this worm is that it can undergo both sexual and asexual reproduction. However, sexual reproduction only occurs in field conditions. When reproducing sexually, worms produce transparent cocoons which contain 4-11 eggs (Drewes & Brinkhurst, 1990). Fertilized eggs then develop into small worms inside the cocoon. When worms are approximately 1 cm in length, they emerge from the cocoon and live freely in the water. Newly hatched worms appear to have a full set of adult-like neurobehavioral responses including peristaltic crawling, giant fiber mediated escape responses to touch and shadow, and helical swimming. These capabilities persist as worms grow to adult size. An adult worm has a body length of about 10 cm and diameter of about 1.5 mm.

In laboratory conditions, worms reproduce exclusively asexually by spontaneous autotomy (fragmentation) into two or more body fragments. Each surviving fragment regenerates head and/or tail segments and grows into a new worm. Under laboratory conditions, worms never reach sexual maturity and usually grow to about 5-6 cm in length.

Lumbriculus, while in sediments or positioned on damp surfaces, crawls by contracting circular and longitudinal muscle layers in each segment (peristaltic crawling). Waves of peristaltic contraction, which begin in anterior segments and travel posteriorly along the body, move the worm forward. Conversely, peristaltic waves, moving from the tail

toward the head, enable the worm to crawl backwards. Such waves of muscle contraction that move the organism in a direction opposite to the direction of body propulsion are called "retrograde waves."

In the "open water," *Lumbriculus* is capable of performing two unique locomotor reflexes: undulatory swimming and body reversal. Undulatory swimming behavior is initiated by touch to posterior segments (Drewes, 1999). Swimming movements consist of series of rapid, rhythmic, rearward waves of helical body bending. Movement of these waves along the body propels the worm forward. Each successive helical wave alternates between clockwise and counter-clockwise orientations. In contrast to swimming, touch to anterior segments evokes a stereotyped body reversal response. The worm responds by rapidly coiling and uncoiling its body resulting in an approximately 180 ° reversal of the body axis (Drewes, 1999).

Circulatory functions in *Lumbriculus* involve a closed circulatory system with red blood that contains a hemoglobin-like pigment, erythrocrucorin. The major blood vessels are dorsal, ventral, and lateral blood vessels. The dorsal blood vessel is pulsatile (Stephenson, 1930). Anterograde pulsation waves in the dorsal blood vessel transport blood from the tail, where oxygenation occurs, throughout the body. Pairs of segmentally arranged lateral blood vessels are also pulsatile and act as auxiliary pumps (Drewes, 1996). In contrast, the ventral blood vessel is non-pulsatile. All blood vessels are easily observed in immature and laboratory cultured worms because the body wall is transparent and unpigmented. Despite these favorable factors, there have been no previous studies relating to basic physiology of blood vessel pulsations in this worm, or any related aquatic oligochaete.

The nervous system of *Lumbriculus variegatus* consists of supra-esophageal ganglion in the first segment and a ventral nerve cord that extends along the body (Stephenson, 1930). One important predator avoidance response in *Lumbriculus* is a rapid escape reflex in which the worm rapidly withdraws its tail or head in response to a threatening stimulus (Drewes & Fournier, 1989). This reflex response is mediated by giant nerve fibers (giant interneurons) located in the ventral nerve cord. Tactile stimulation of the worm's body initiates impulses in the giant nerve fibers. Impulses in giant nerve fibers trigger motor neurons which in turn send impulses to the longitudinal muscle, resulting in body shortening (Zoran & Drewes, 1987; Drewes & Fournier, 1989; Drewes & Brinkhurst, 1990). All-or-none action potentials in giant nerve fibers of *Lumbriculus* can be recorded using non-invasive electrophysiological recording methods (Rogge & Drewes, 1993) which offers potential for long-term analysis of change in giant fiber function with respect to development and regeneration.

When worms are seized by predatory attack, segments may be injured or amputated. The possibility that worms actively autotomize injured segments has not been investigated in this organism or any other annelids. Following amputation, surviving fragments are faced with the same challenge as autotomized fragments during asexual reproduction. The way the worm meets this challenge is by segmental regeneration. This regeneration process begins with blastema and bud formation followed by differentiation of numerous new head and tail segments. These newly regenerated segments undergo *de novo* synthesis of the central nervous system, peripheral nervous system, body wall muscle, and other tissues. In addition, the newly formed central nervous system must establish proper interconnections with the pre-existing central nervous system. Because new connections are established between new and old segments during regeneration, we might expect that reconnections would also be readily

made in cases of transected and ablated ventral nerve cord. However, overcoming the problem of surgery-induced autotomy responses is a major obstacle to performing such studies.

Though most segments in worms appear superficially similar to each other in external morphology, there are significant differences in structure and function of segments. These differences appear as systematic longitudinal gradients with respect to microanatomy, physiology, and behavior. An example of such gradients is giant nerve fiber diameter. The medial giant fiber decreases in diameter along the body from anterior to posterior. Conversely, lateral giant fibers increase in diameter from anterior to posterior (Zoran & Drewes, 1987). Most other organ system in the worm also display axial gradients in structure and function.

Dissertation Objectives

My work is presented as four papers, all representing novel approaches to physiology, behavior, and development of *Lumbriculus variegatus*. A unifying theme is description of the axial gradients, especially as they relate to several biological processes: giant nerve fiber regeneration, locomotion, reflex behaviors, segmental autotomy, and dorsal blood vessel pulsation.

Chapter 2 of this dissertation is an investigation and characterization of a novel autotomy reflex in *Lumbriculus*. This is the first detailed description of such a reflex in any annelid worm. Longitudinal and developmental gradients with respect to the propensity to autotomize were examined. Autotomy is an active process which can be induced by body

compression. In addition, it was discovered that nicotine can be used as a powerful blocker of autotomy (Lesiuk & Drewes, 1999).

Chapter 3 is a study of neural regeneration in *Lumbriculus*. In this chapter, we used nicotine as a tool to immobilize worms and block the autotomy process. This allowed surgical access to the ventral nerve cord via a ventral incision and transection of the worm's ventral nerve cord. Thus, all experiments can be done *in vivo* and repeated measurements in the same worm can be made throughout recovery. The main focus of this study was a characterization of the time-table of regeneration of central conduction pathways of the ventral nerve cord. We specifically looked at locomotor reflexes, electrophysiological recordings, and morphology of the regenerating ventral nerve cord.

Chapter 4 is a study involving more invasive and more radical surgical manipulation in *Lumbriculus*. The surgery was done by removing 5-8 segments of ventral nerve cord (= ablation). The time-table of regeneration and the induction of ectopic outgrowth were studied using the same procedures as in transection experiments. When the ventral nerve cord was ablated, some worms regenerated an ectopic head at the point of surgery. The influence of the ectopic head on axial gradients of *Lumbriculus* was studied in relation to locomotor reflexes, electrophysiological recordings, and histology. This chapter was done as a companion paper to chapter 3 to be submitted to Invertebrate Zoology or Invertebrate Reproduction and Development.

Chapter 5 of this dissertation involved the development of simple and reliable laboratory methods for rapid quantitation of *in vivo* basal pulsation rates in *Lumbriculus*. Longitudinal gradients with respect to pulsation rates in the dorsal blood vessel were also examined. Characterization of *in vivo* effects of two neuropharmacological agents, nicotine

and caffeine, were reported. This section has significant potential for both educational and research applications and was published in American Biology Teacher.

Dissertation Organization

The dissertation is composed in the alternate format. Chapters 2-5 of this dissertation is a complete manuscript modified to conform to the specifications of the Iowa State University Thesis Office. In each chapter, there is an introduction, material and methods, results, discussion, and references. A general introduction precedes Chapter 2 and there is a general summary of the dissertation following the Chapter 5.

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**CHAPTER 2. AUTOTOMY REFLEX IN A FRESHWATER OLIGOCHAETE,
Lumbriculus variegatus (CLITELLATA: LUMBRICULIDAE)**

A paper published in *Hydrobiologia*

Nalena M. Lesiuk & Charles D. Drewes

Abstract

A novel apparatus was developed that induced segmental autotomy in the freshwater oligochaete, *Lumbriculus variegatus*. The apparatus delivered a quantifiable amount of focal compression to the dorsal body surface at a selected site along the worm. This resulted in a rapid and stereotyped autotomy sequence, beginning with formation of a lateral fissure in the body just anterior to the compression site. Formation of the fissure usually occurred 100-200 ms after the onset of compression. Autotomy readily occurred in the absence of significant longitudinal tension at the autotomy site and in the absence of direct laceration of the body wall. Autotomy culminated in a complete, transverse separation and sealing of anterior and posterior body fragments with no apparent blood loss from either end. There was a direct relationship between the amount of compression and the probability of autotomy in both midbody and tail regions. However, there was a consistently greater probability of autotomy in tail versus midbody regions. Autotomy did not occur if the duration of compression was less than 77 ms. Autotomy responses were suppressed in concentration-dependent manner by a 15 min treatment with nicotine prior to compression. In instances where compression just failed to induce autotomy there was no evidence of disruption of impulse conduction in giant nerve fibers. Rapid and clear-cut autotomy, in combination with this worm's significant

capacity for regeneration of lost segments, are adaptively significant strategies for surviving predatory attack.

Keywords: autotomy, fragmentation, reflexes, nicotine, lumbriculid, blackworms

Introduction

Autotomy is a well known behavior among a variety of animal groups such as arthropods (Foelix, 1996), echinoderms (Emson & Wilkie, 1980) and vertebrates (Goss, 1969).

Generally, autotomy responses are triggered by direct damage or encounter with threatening stimuli. The result is a stereotyped effector response leading to rapid separation and discarding of a body part (such as an appendage) from the main body. An autotomized body part may serve as a possible sacrifice or a source of distraction to a predator. The latter is especially true in cases where appendages generate substantial movements after autotomy (Arnold, 1990).

Invertebrates without appendages, such as annelid worms, are known to autotomize body segments in response to injury from mechanical or chemical stimuli (Stephenson, 1930; Cameron, 1932; Rasmussen, 1953). In some earthworm species, only anterior fragments are viable and provide substrates for subsequent regeneration of missing segments (Cameron, 1932). However, in a few cases (e.g., the freshwater oligochaete, *Lumbriculus variegatus* Grube 1884, and marine polychaete, *Pygospio elegans* Claparède 1863) autotomy results in small body fragments that survive and readily regenerate missing head and/or tail segments, thus forming new individuals (Stephenson, 1930; Rasmussen, 1953). Despite propensities for segmental autotomy in at least a few species, there has been no previous analysis, in any annelid, of the mechanisms of segmental autotomy and its behavioral correlates.

Here, we tested the hypothesis that segmental autotomy in a freshwater lumbricolid, *L. variegatus*, is a stereotyped and reflexive motor response which may be consistently induced by adequate stimulation in the form of sudden body compression. Our hypothesis derives from a previous report which noted that these worms are prone to segmental autotomy. This occurred inadvertently while worms were handled and manipulated in preparation for electrophysiological studies of segmental regeneration (Drewes & Fournier, 1990). Our hypothesis is predicated on the assumption that mechanical compression of the body is a relevant stimulus because living worms may be subject to such stimuli during predatory attack (e.g., pinching or biting) in their littoral environment. After developing and testing an apparatus that delivers precisely controlled amounts of body compression to individual worms, we used frame-by-frame video analysis to examine the timing of autotomy in relation to the amount and duration of body compression. We also examined electrophysiological correlates of ventral nerve cord function in instances in which stimuli just failed to elicit autotomy. Finally, we demonstrated pharmacological blockade of the autotomy response by exposure to nicotine, a cholinergic agonist.

Materials and methods

Animals

Lumbriculus variegatus (blackworms), ranging in size from newly hatched to sexually mature adult, were used. Worms were obtained from a variety of sources depending on size; four different size categories were selected.

Mid-sized worms, used for most experiments, were obtained from asexually reproducing laboratory cultures. These were raised in aged (dechlorinated) tap water using

brown paper towel as a substrate. Worms were fed twice weekly with sinking fish food pellets. Cultures were aerated and water temperature was held at 22-23°C. Body diameter (at mid-body) and overall length were about 520 μm and 3.5 cm, respectively. These values represent the approximate upper limit in body size attained before spontaneous fragmentation (asexual reproduction) occurred in the worms in our laboratory cultures. Mid-sized worms were pre-screened for uniformity in segment size and pigmentation. Worms with abrupt discontinuities in segment size and pigmentation were not used; typically such discontinuities are indicative of very recent tail segment regeneration.

Larger, sub-adult worms were obtained from a local pet shop which receives weekly shipments of field-collected worms originating near Fresno, California. Body diameter (at mid-body) and length of these worms were about 900 μm and 5 cm, respectively.

Sexually mature (adult) worms were obtained in May from a marsh in Gull Point State Park (West Lake Okoboji, Iowa). Body diameter (at mid-body) and lengths of these worms were 1150 μm and 8 cm, respectively.

Newly hatched worms were obtained from cocoons collected from the same site as adult worms. Transparent cocoons, about 5 mm in length and containing 4-12 orange eggs, were found attached to submerged leaf litter. After approximately one week of development in the laboratory (22-23°C), worms emerged from cocoons. Segment diameter (at mid-body) and worm length were about 170 μm and 0.5 cm, respectively.

Apparatus for Inducing Autotomy

We designed a novel apparatus for delivering a brief mechanical stimulus that reliably induced segmental autotomy in individual worms (Fig. 1). The stimulus consisted of localized compression caused by rapid application of focal pressure, in a transverse

orientation, to the dorsal surface of the body. Compression was caused by direct pressure to the dorsal body wall with the smooth, flattened edge of an aluminum "blade" (blade thickness = 0.27 mm). The blade was attached to a pintle which permitted a pendulum-like movement of the blade, as shown in Fig. 1. The holder for the pintle was attached by a short rod to the axial post of a pen driver motor (originally from a strip chart recorder).

Rectangular pulses from a Tektronix FG 501 wave function generator provided direct electrical input to the pen motor. The output of the function generator was, in turn, controlled by the square-pulse output from a Grass SD9 stimulator (Fig. 1). When the single-pulse switch of the stimulator was pressed, a stimulus pulse (20 ms duration) was routed to the gating input of the function generator. This resulted in production of exactly one cycle of rectangular wave output from the function generator. The cycle duration of this wave, was controlled by the frequency control on the function generator. A low frequency value (= long cycle time) resulted in a compression stimulus with long duration. In most experiments the cycle duration was adjusted to 5 s. In some experiments, cycle duration was varied between 65 ms and 5 s. Exact measures of compression durations were made by placing a piezoelectric disk beneath the blade. Transient outputs from the disk indicated the timing of blade contact and release, as measured on an oscilloscope.

The output pulse from the function generator resulted in axial rotation of the pen motor shaft and 0.6 cm downward excursion of the blade. Blade movement was limited by its contact with the flat surface of a brass annulus (outside diameter = 2.4 cm; inside diameter = 0.7 cm). As the blade contacted the annulus, the pintle to which the blade was attached rotated slightly. This compensated for the slight curvilinear motion of the blade and assured that blade contact with the annulus was flush with each compression stimulus. The center

space of the brass annulus was occupied by the optically flat end of the movable post from an electronic digital micrometer (Fred V. Fowler Co., Inc.).

Prior to delivery of the compression stimulus, the micrometer post was set exactly level with the surface of the annulus and the zero button on the micrometer was pressed. Then, the micrometer post was adjusted to the desired distance below the level of the annulus, according to the digital readout on the micrometer (readout to nearest μm). This distance was equal to the desired height of the body, under the blade, at the time of compression. For example, if the desired compression level was 90% for a worm, whose segment diameter was $1000\ \mu\text{m}$, then the micrometer post was correspondingly set to $100\ \mu\text{m}$ below the level of the annulus. Micrometer settings were recalculated with each compression stimulus according to the worm diameter and desired compression value (Fig. 2). Once calculations and appropriate micrometer settings were made, a worm was transferred along with a small amount of water to the autotomy platform. A fine glass hook was used to position the worm so that segments from a specified location rested across the micrometer post and the body axis was perpendicular to the blade above.

Then, the single-pulse switch on the stimulator was manually pressed, resulting in immediate downward movement of the blade. Blade velocity at the time of contact with the worm was $0.66\ \text{m/s}$, as measured by electronic sensors. Blade velocity remained constant, regardless of changes in the duration of the compression stimulus.

Autotomy responses to the compression stimulus were recorded on video tape using a JVC Model TK 1070U color video camera and Model HRS7300U video cassette recorder. The camera was mounted on an Olympus Model SZ CTV dissecting microscope. Camera frame rate was 30 frames/s. Camera shutter-speed was adjusted to $1/500\ \text{s}$ and the autotomy

platform was illuminated with a fiber optic illuminator. This combination of equipment and specifications insured relatively clear, freeze-frame images of worms immediately before and after contact with the blade. Video images were electronically captured and then photographed with a SONY UP1200A video printer.

Nicotine Preparation

Nicotine solutions were made with double distilled water the day of use. Worms were exposed via immersion in a 20-ml aliquot of nicotine solution in a glass container (one worm/container). Based on preliminary range-finding experiments, the following nicotine concentrations and exposure time were selected for final experiments: 0.002-0.250 mM nicotine and 15 min exposure. After nicotine exposure, worms were immediately rinsed with double distilled water and placed on the autotomy chamber.

Electrophysiology

Worms that did not autotomize in response to specific amounts of compression were electrophysiologically tested to determine whether the velocity of impulse conduction in giant nerve fibers was affected by the compression stimulus. All-or-none action potentials from the medial and lateral giant nerve fibers in the ventral nerve cord were recorded non-invasively, as previously described (Drewes & Fournier, 1990; Rogge & Drewes 1993). The worm was placed on a printed circuit board recording grid. Giant fiber conduction velocity was then measured immediately before compression, over a 1 cm distance at a midbody position. Medial and lateral giant fiber spikes were evoked by light tactile stimulation to the worms' head or tail, respectively, using a hand-held probe. Amplified signals were displayed and measured using a digital storage oscilloscope. The same protocols were used when

measuring conduction velocity immediately after the compression, making sure that the compression site was positioned between the two pairs of recording electrodes.

In non-autotomized worms that were pre-treated with nicotine, measurements of the conduction velocity were made 4 h after the compression stimulus, a time that was sufficient for recovery from nicotine effects on escape reflex pathways and function. During recovery, worms were placed in aged tap water. Conduction velocity values after recovery were then compared to those from a corresponding location before nicotine treatment and compression.

Results

Videotape analysis provided direct support for our hypothesis that autotomy is a reflexive response which is consistently induced by brief compression of the body. This response consists of a stereotyped sequence of mechanical and behavioral events. Initially, full excursion of the blade (from fully up to fully down position) occurred in less than one video frame (<33 ms). Once the blade was in the fully down position, body segments under the blade (approximately 1-2 segments in mid-sized worms) were distended laterally by the pressure but these segments were not lacerated or ruptured. Segments immediately adjacent to the compression point, on both sides of the blade, bulged slightly (Fig. 3A). Then, during the next several frames, one or several lateral bending movements occurred anterior to the compression point. Similar movements, though slower and weaker, also occurred in segments posterior to the compression.

After this, one of two results occurred: successful autotomy or failure to autotomize. That is, autotomy appeared to be an all-or-none process in which segments quickly and cleanly separated from one another near the point of compression or remained fully

connected and intact. There were never instances in which autotomy was incomplete or segments remained partially attached to one another.

In cases of successful autotomy, a transverse fissure formed along one side of the body wall. Invariably, this fissure occurred just anterior to the compression point, at a site approximately midway between the anterior and posterior segmental groove of one segment. Then, during the next few frames, the fissure became circumferential, anterior and posterior portions separated, and autotomy was completed (Fig. 3A). Importantly, at the instant of separation, there was little or no pulling back or recoil of the free anterior portion of the worm away from the blade (Fig. 3A, frame f7). This indicates that there was little or no longitudinal tension exerted by the anterior end upon the compression point. Given the experimental conditions, establishment of such tension would be necessarily limited and unexpected due to the worms' inability to obtain traction against the smooth metal surfaces on the post and annulus. After autotomy was completed, but before the termination of compression, the posterior fragment remained briefly trapped under the blade until the blade lifted. Video images at the time of autotomy showed no indication of blood leakage from anterior or posterior ends of autotomized segments.

Immediately after autotomy, a rapid circumferential closure of the body wall occurred, resulting from a crimping of the terminal segment of each fragment. By 10 min after autotomy, the exposed terminal surfaces on both anterior and posterior fragments appeared rounded and smooth (Fig. 3B). However, the first one or two segments of the posterior fragment were pale and thinner in diameter than the rest of the fragment. The unusual appearance of these segments was apparently related to their brief entrapment under

the blade, as previously described. Pulsations in the dorsal blood vessel of these pale segments were re-established approximately 20 min after autotomy.

In all worms that failed to autotomize in response to compression, there was no apparent laceration or rupture of the body wall after the blade lifted, although segments under the blade were clearly affected by the compression. These segments were pale and thinner in diameter than neighboring segments (Fig. 3C). Gradually, over the next few hours, segment diameter appeared normal and dorsal blood vessel pulsations were re-established throughout these segments. There was no sign of blood vessel rupture or internal hemorrhage after compression.

To further test for after-effects of compression in non-autotomized worms, the functional integrity of the nervous system was assessed by non-invasive neurophysiological recording of giant nerve fiber spikes. Sub-adult worms were electrophysiologically tested before and immediately after a compression stimulus (85%). The conduction velocities of medial and lateral giant nerve fibers were not significantly changed by compression. The average medial giant fiber conduction velocity before compression was 11.4 ± 0.9 SD m/s; immediately after compression, it was 11.2 ± 0.7 SD m/s. Similarly, the average lateral giant fiber conduction velocity was 7.3 ± 0.5 SD m/s before compression and 7.3 ± 0.5 SD m/s after. Thus, compression stimuli caused no significant alteration in the conduction properties of nerve fibers.

Next, quantitation of the timing of the autotomy response was done using frame-by-frame analysis of video images. Time taken to autotomize was determined by counting the number of elapsed frames between the first frame in which the blade was down and the first frame in which a lateral fissure of the body wall was evident (= onset of autotomy). Figure 4

shows a compilation the elapsed times from compression to onset of autotomy in 50 mid-sized worms. The median and mode of this distribution were both 133 ms (four video frames). Autotomy onset occurred at this time in twelve of 50 worms. Nine worms required more than 200 ms to autotomize, thus slightly skewing the distribution to the right. The shortest time for autotomy onset was 67 ms (two video frames); four of 50 worms autotomized at this time.

These results raise questions about the minimal stimulus duration required for autotomy. To test this, the duration of compression, as set on the stimulator (Fig. 1), was varied from 65-540 ms. The level of compression was set at 95%, regardless of duration. No worms autotomized when durations were set to 65 ms ($n = 20$) and 77 ($n = 20$) ms. Two of 20 worms autotomized when the duration was 91 ms. The percent autotomy increased as the duration increased, until 100% (20 of 20) autotomized when the duration was 540 ms (approximately 16 frames). These results demonstrate that there is a lower limit in the duration of the stimulus compression required for autotomy; this limit was approximately 65-77 ms.

Effect of Amount and Location of Body Compression

We next tested the possibility that there is a threshold stimulus (i.e., critical amount of compression) for autotomy induction and that this threshold differs in relation to the locus of compression. Figure 5 shows the relationship between changes in percent autotomy and amount of compression in mid-sized worms. This graph shows a direct effect of the amount of compression on percentage of autotomy. In a midbody location where body diameter is approximately 520 μm , about 30% of worms autotomized when the compression was 90%. However, all worms autotomized with 90 to 95% compression.

For studying compression effects in posterior segments, micrometer settings were adjusted to compensate for the relatively smaller segment diameter (approximately 430 μm). As with midbody compression, autotomy increased as the compression in the tail region increased. However, some worms (36%) autotomized at a compression value of 85%. When compression was increased to 90%, most worms autotomized (71%). Further increase in compression to 95% resulted in 100% autotomy. Thus, autotomy responses were more easily induced in tail than midbody regions.

Nicotine effects

Since chemical synaptic transmission in annelid neuromuscular junctions, and in at least some central synapses, is cholinergic (Gerschenfeld, 1973), we examined the possibility that the cholinergic agonist, nicotine, may somehow disrupt autotomy effector responses.

Nicotine effects on autotomy were tested in mid-sized worms using a 15 min treatment prior to compression. A value of 95% compression was selected for all treated worms since this concentration normally results in 100% autotomy. There was a dose-dependent reduction in autotomy in relation to increased nicotine concentration (Fig. 6). This trend was clearly evident for both midbody and tail regions. When nicotine concentration was higher than 0.01 mM, no worms autotomized in response to midbody compression. Likewise, autotomy was suppressed by nicotine in the tail region, where only 5% autotomized when exposed to 0.01 mM and none with 0.1 mM nicotine.

The question then arose as to whether nervous system function in non-autotomized, nicotine-treated worms was affected by 95% compression. A group of worms was selected for non-invasive electrophysiological testing before and after compression. The average medial giant fiber conduction velocity, before nicotine treatment and compression, was $8.3 \pm$

0.6 SD m/s. After recovery from nicotine (4 h), velocity was 8.1 ± 0.8 SD m/s. Similarly, the lateral giant fiber conduction velocity was 5.5 ± 0.8 SD m/s before treatment and compression, and 5.2 ± 0.6 SD m/s after recovery. Neither of these changes was statistically significant. Thus, there was no sign of functional damage to giant nerve fibers in worms following nicotine treatment and 95% compression.

Size effect

Additional experiments were done to compare tendencies for autotomy in newly hatched, mid-sized, subadult, and adult worms in response to a 90% compression stimulus. Autotomy occurred in 57% ($n = 7$) of newly hatched, 71% ($n = 50$) of mid-sized, 80% ($n = 20$) of subadult and 80% ($n = 10$) of adult worms. Videotape analysis revealed that the timing and appearance of the autotomy responses in all of these stages were similar to those shown in Fig. 3.

Discussion

We have developed an apparatus for reliably inducing and quantifying segmental autotomy in *L. variegatus*. Our results indicate that autotomy is a rapid response that may be reflexively triggered by sudden application of mechanical stimulation. Particularly effective stimulus parameters included a combination of focal application of transverse body compression at a level of at least 85% (see Fig. 2) and a duration of at least 65 ms. These stimuli were severe enough to cause some overt trauma to segments but not severe enough to directly lacerate the body wall or disrupt functional integrity of the ventral nerve cord. With adequate stimulation, autotomy was initiated by the worm itself as a stereotyped, all-or-none response in which the body is cleanly and quickly separated into two fragments.

Since any segment in mid-body or tail regions appeared capable of initiating autotomy in all body sizes, it seems reasonable to hypothesize that sensory and effector elements involved in autotomy are metamerically arranged and present at all stages of postembryonic growth. These results raise questions regarding the reflex mechanisms of autotomy. Are there mechanosensory neurons that specifically detect compression or other traumatizing stimuli? What muscles or other effector structures are involved in quickly separating and sealing off the ends of body fragments? The rapid formation of a lateral fissure and the clean separation of anterior and posterior fragments (Fig. 3) suggests that each segment may contain a pre-formed, circumferential autotomy site, perhaps comparable to the autotomy planes seen in the limbs of several arthropod groups (Bliss, 1960; Needham, 1965; Foelix, 1996). Investigations are currently underway to examine effector mechanisms involved in autotomy, as well as possible similarities between stimulus-induced autotomy and spontaneous self-fragmentation that occurs in asexually reproducing cultures in this species.

The capability of initiating rapid autotomy in response to mechanical trauma likely provide numerous survival advantages in relation to predatory pressures that undoubtedly exist in the worm's littoral habitat. First, rapid autotomy could enhance the probability of escape and survival of one or both worm fragments. Since the site of autotomy is just anterior to the site of body compression, anterior segments would be more likely to be spared if the predatory attack involved a bite or pinch at mid-body or posterior locations. Second, the rapid sealing of the body wall could minimize the potential chemo-attractiveness of autotomized segments to predators. Rapid sealing could also reduce blood loss, osmotic stress, and necrosis in segments adjacent to the autotomy plane. These factors, in

combination with the consistent formation of a circumferential autotomy plane, would help to normalize the subsequent processes of blastema formation and segment regeneration.

The pharmacological effects of nicotine on responses to compression (Fig. 6) raise the possibility that some controlling step(s) in the autotomy reflex pathway may utilize nicotinic cholinergic receptors, but the specific site and mechanism of nicotine action are unknown. We are currently using nicotine as a pharmacological tool (paralytic agent) for routinely blocking the autotomy response. Circumvention of autotomy by nicotine treatment is especially useful because it allows surgical invasion and manipulation of the central nervous system, thus introducing significant opportunity for studying processes of neural repair and regeneration.

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Figure legends

Fig. 1. Diagram of apparatus for inducing autotomy. Abbreviations: ST (stimulator);

WG (wave function generator); MO (motor); B (blade); P (micrometer post); A (circular brass annulus); D (dish).

Fig. 2. Quantitation of compression stimulus. A) The normal cross-sectional height of the body (H_n) is shown before transverse compression with the narrow blade. B) The compression height of the body (H_c) is shown with the blade resting on the annulus. The formula for % compression is given below.

Fig. 3. Freeze-frame video images of posterior segments during and after body compression. In A, panel f1 shows the first frame in which the blade is lowered and the body is compressed. The location of the blade is shown by the dots. Panels f3, f5, and f6 show the weak lateral bending movements that precede autotomy. Separation of the anterior portion end of the worm is shown in f7. Panels B1 and B2 show the rounded and sealed ends of the anterior body fragment and posterior body fragment, respectively, 10 min after autotomy. Panel C shows the appearance of body segments immediately after 85% compression (delivered at asterisk), which failed to trigger autotomy.

Fig. 4. Histogram showing elapse time from delivery of compression stimulus to onset of autotomy in 50 worms. Each bar indicates the number of worms whose autotomy onsets correspond to the number of video frames. The shortest time for autotomy onset was two video frames. Median and mode of this distribution were four video frames, corresponding to an elapsed time of 133 ms.

Fig. 5. Graph showing relationship between compression amount and percent autotomy for

two locations along the worm. Each point is a mean for 50 worms.

Fig. 6. Dose-response effects of nicotine on autotomy. Each point indicates the percentage of worms (20 worms/ point) that autotomized at a particular concentration and body location.

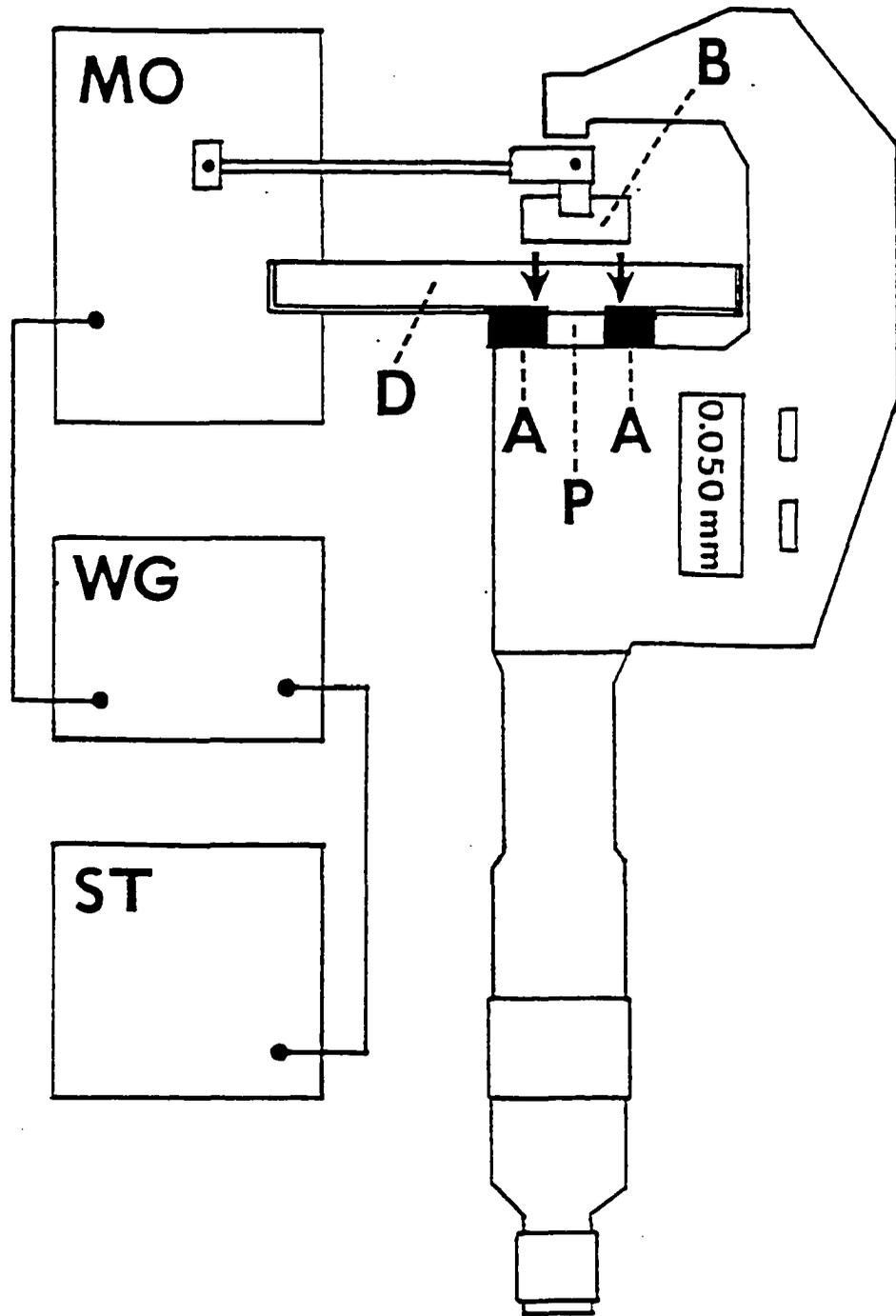
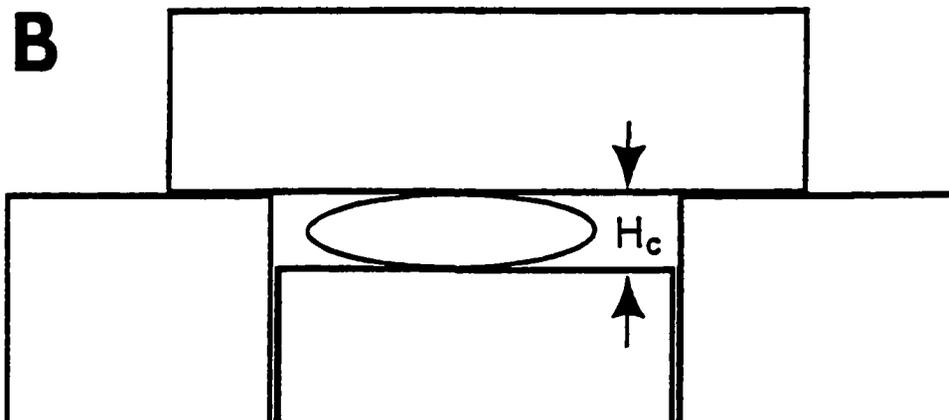
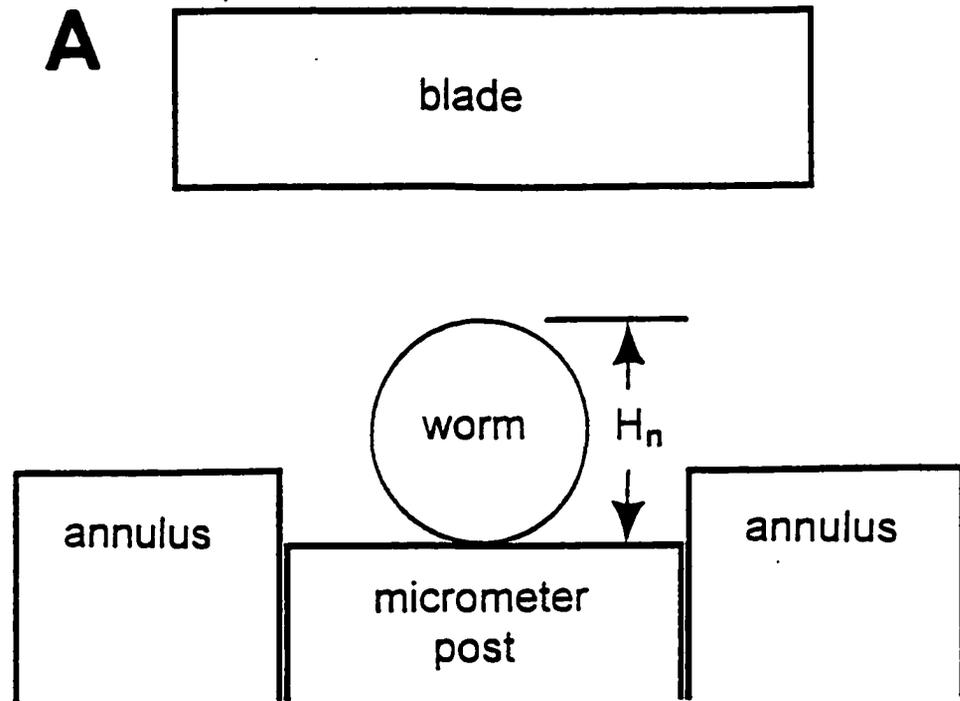


Figure 1.



$$100 - (H_c / H_n \times 100) = \% \text{ compression}$$

Figure 2.

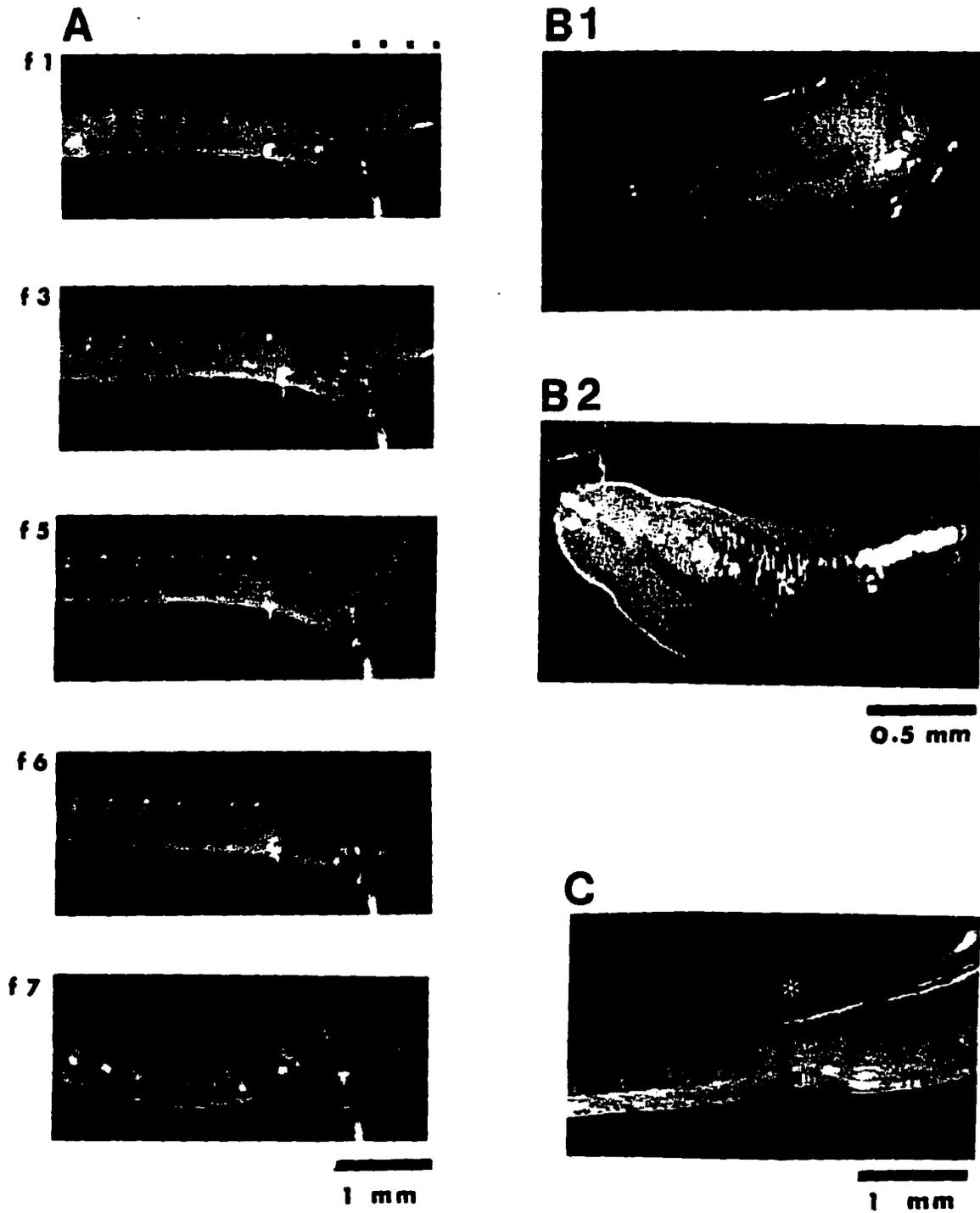


Figure 3.

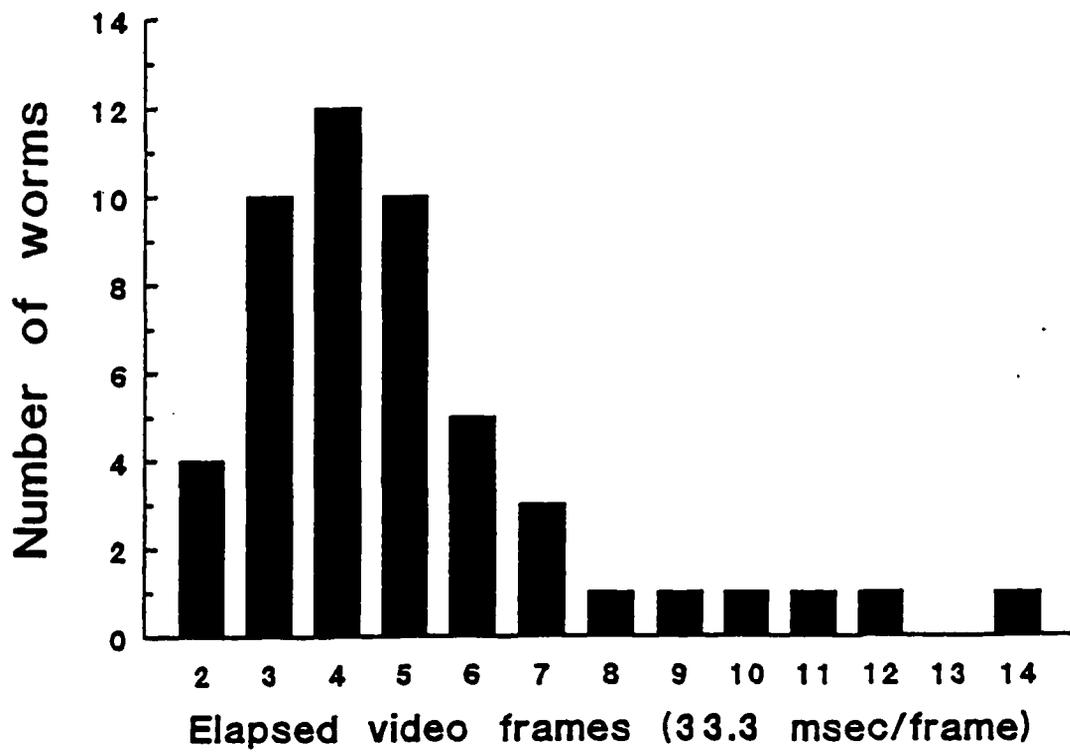


Figure 4.

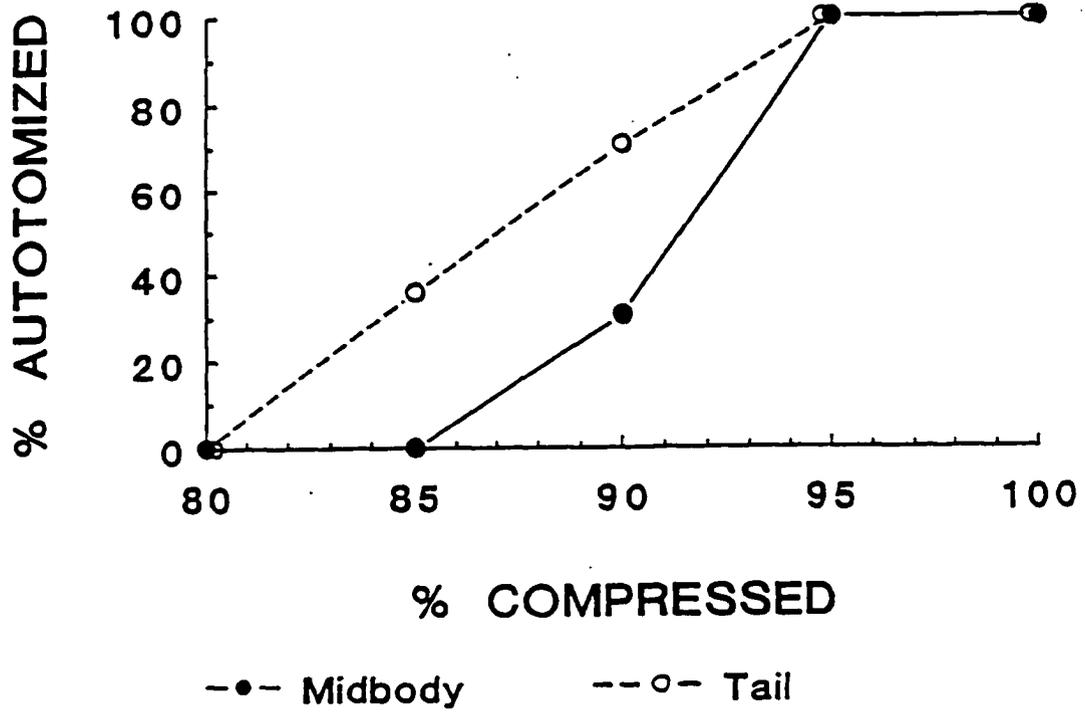


Figure 5.

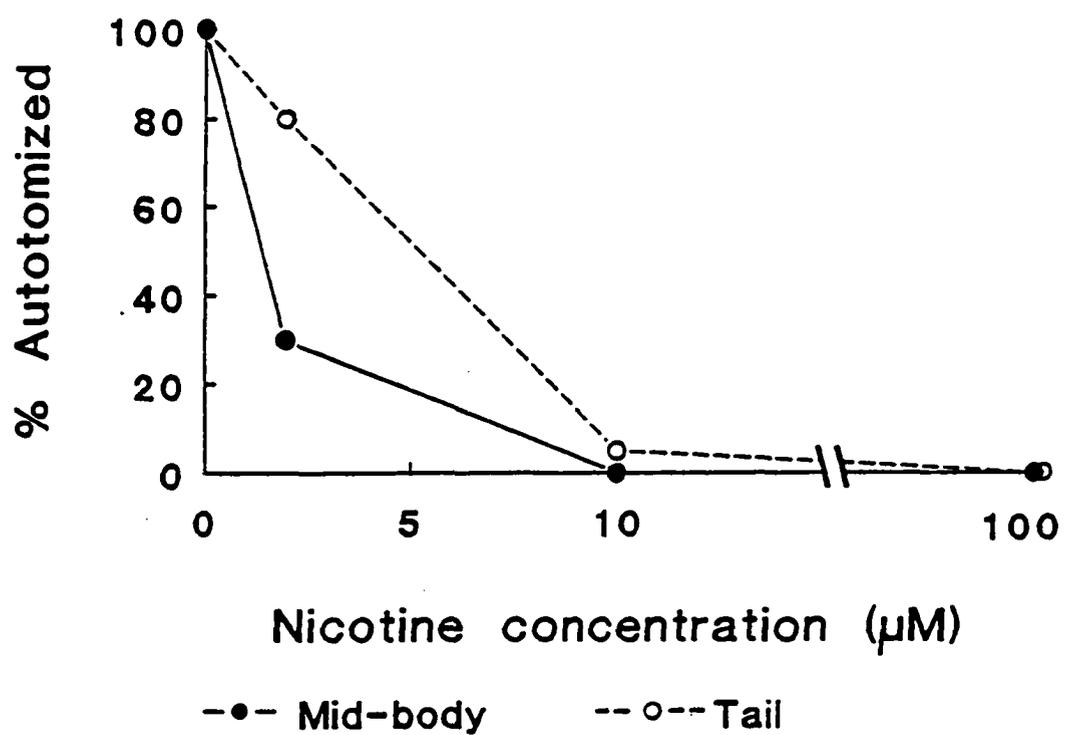


Figure 6.

CHAPTER 3. BEHAVIORAL PLASTICITY AND CENTRAL REGENERATION OF LOCOMOTOR REFLEXES IN THE FRESHWATER OLIGOCHAETE, *Lumbriculus variegatus*. I: TRANSECTION STUDIES

A paper to be submitted to Journal of Invertebrate Biology

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Abstract

Regeneration of central nervous system, after ventral nerve cord transection, was studied in an aquatic oligochaete, *Lumbriculus variegatus*. Normally, such experimental manipulation of the worm's ventral nerve cord is impossible because surgical invasion induces segmental autotomy (self-fragmentation of the body). However, results here showed that nicotine is a powerful paralytic agent that immobilized the worm, blocked segmental autotomy, and allowed neural surgery. Analysis of non-invasive electrophysiological recordings and behavioral responses were used to characterize a timetable of functional recovery of giant nerve fibers and other reflex pathways in worms with a transected ventral nerve cord. During the first several hours after transection, medial giant fiber (MGF) or lateral giant fiber (LGF) spikes were conducted up to, but not across, the transection site. For both MGF and LGF, the first sign of reestablishment of coupling of spikes across the transection site occurred as early as 10 h (and usually within 20 h after transection). Initially, the conduction times for both MGF and LGF, as measured over a 6 mm distance that included the transection site, were approximately 50% longer than before transection. This delay in conduction was significantly decreased to a near normal value within 20 h after transection. An analysis of non-giant-mediated behavioral responses was also made in association with ventral nerve cord transection and subsequent regeneration. Immediately after ventral nerve cord

transection, there was a functional reorganization in touch-evoked locomotor reflexes, such that the two portions of the worm, anterior and posterior to the transection site, were independently capable of initiating swimming and body reversal responses. A similar reorganization of behavior responses occurred in amputated body fragments. A reversion back to the original whole-body pattern of swimming and reversal responses was the first sign of central regeneration following ventral nerve cord transection. This occurred as early as 8 h after transection. Thus, functional restoration of the non-giant fiber pathways appeared slightly faster than that of giant fiber pathways. The results demonstrate the remarkable plasticity of locomotor reflex behavior immediately after ventral nerve cord transection or segment amputation. They also demonstrate the exceptional speed and specificity of regeneration in central pathways that mediate locomotor reflexes in these worms.

Additional keywords: giant nerve fibers, locomotion, reflexes, regeneration, neural plasticity

Introduction

The ventral nerve cord in some species of oligochaete worms has a high capacity for regenerative repair (Drewes et al., 1988). The main pathways in which regeneration has been investigated are the intersegmental giant interneurons whose enlarged axons form a rapid, through-conduction pathway that reflexively mediates rapid escape withdrawal.

Regeneration of oligochaete giant nerve fibers has been most extensively studied in terrestrial earthworms. In the earthworm, *Eisenia foetida* (Vining & Drewes, 1982; Vining, 1984), the apposed cut ends of the transected ventral nerve cord form outgrowths containing

sprouts arising from medial (MGF) and lateral giant fibers (LGF). The anatomical reconnections between these outgrowths typically occur about 24-48 h after transection. Such anatomical coupling is accompanied by functional recovery of through-conduction across the transection, as indicated by electrophysiological recordings of giant nerve fiber spikes from intact worms. In larger earthworm species, such as *Lumbricus terrestris*, recovery is slower, requiring about 5-10 d and 12-18 d for restoration of through-conduction in MGF and LGF, respectively (Balter et al., 1980).

The regenerative capabilities of the ventral nerve cord in the aquatic counterparts of earthworms (i.e., freshwater oligochaetes) have not been studied. Such studies present certain challenges because of the small size of these worms and, in some cases, the tendency for segmental autotomy (body fragmentation) in response to surgical invasion. Nevertheless these worms present features that appear favorable for such study. These features include a high capacity for segment regeneration and *de novo* formation of the ventral nerve cord (Morgulis, 1907; Stephenson, 1936), as well as the capacity for morphallactic transformation of the nervous system in regenerating body fragments (Drewes & Fournier, 1990). In addition, these worms are highly amenable for studying electrophysiological properties of giant nerve fibers *in vivo* using non-invasive electrical recordings (Zoran & Drewes, 1987).

The overall objective here was to study regeneration capacity in the freshwater oligochaete, *Lumbriculus variegatus* (Order Lumbriculida) following ventral nerve cord transection. Specific objectives were: (1) to develop protocols that circumvent the problem of segmental autotomy so that surgical invasion and transection could be routinely done; (2) to characterize, on an hour-by-hour basis, the timetable of functional recovery of giant nerve fibers using non-invasive electrophysiological recording methods; (3) to assess functional

recovery of two non-giant-mediated locomotor responses, helical swimming and body reversal. These novel behaviors, possessed by a few species of aquatic oligochaete, were recently described by Drewes (1999).

Material and Methods

Animals

Freshwater oligochaetes, *Lumbriculus variegatus*, were obtained from laboratory cultures. In culture, these worms do not reach the body size required for sexual maturity (about 9-10 cm length). Instead, they usually attain a length ranging from 5-6 cm and then undergo fragmentation (asexual reproduction).

Worms were maintained in artificial sediments (strips of brown paper towel) and dechlorinated tap water. They were fed sinking fish food several times weekly and kept at room temperature (23 °C).

Only worms showing no abrupt discontinuity in segmental dimensions were selected for experiments. Such discontinuities are indicative of recent segmental regeneration. Before transection or testing, worms were isolated from cultures and placed overnight in a container of fresh spring water to allow clearance of intestinal contents.

Prior to transection, worms were immobilized by placement in 20 ml of 0.25 mM nicotine for 15 min. Immediately after removal, worms were fully immobilized and relaxed. In this state, the overall resting length of worms was slightly greater than in normal worms. Also at this time, the dorsal and lateral blood vessels of the worm were still rhythmically pulsating, but there were no detectable spontaneous body movements or reflex responses to touch stimuli.

Pilot studies were done in a separate group of worms to test the duration and reversibility of nicotine effects. After removal from nicotine, worms in this test group were placed in fresh spring water, where they remained in an immobilized state for approximately 20 min. At this time, some signs of gradual recovery were evident, especially weak spontaneous movements in anterior segments. Full recovery from nicotine effects occurred within 3 h, as indicated by restoration of normal locomotor behaviors and normal electrophysiological responses to touch.

In experimental groups, worms were exposed for 15 min to the 0.25 mM nicotine solution, as previously described. Then, they were rinsed briefly and transferred to a silicone rubber dish. They were positioned ventral side up and pinned to the dish with two minuten insect pins (stainless steel; 0.05 mm diameter). The two pins were placed approximately 5 mm apart so that the desired site of transection was midway between the pins. When pinning the worms, special efforts were made to avoid impalement of the ventral nerve cord, which was clearly visible through the ventral body wall. In all cases, the selected transection site was in the posterior half of the worm, as shown in Fig. 1 and Fig. 2.

A short, shallow, transverse incision through the body wall was made in one segment, midway between two adjacent segmental boundaries, using the tips of a microdissecting scissors (Vannas style iris spring scissors, 3 mm cutting edge). The incision was about 0.2 mm in length, or approximately twice the diameter of the ventral nerve cord. Another minuten pin was then inserted transversely under the exposed ventral nerve cord in order to separate the cord from underlying tissues.

The tips of the microdissecting scissors were then placed on one side of the pin and the ventral nerve cord was completely severed. At this time, the two cut ends of the ventral

nerve cord separated by about 0.2-0.3 mm (~ the length of one body segment). Precautions were made to avoid cutting the ventral blood vessel and intestine. No sutures or other procedures were used to close or treat the wound after the transection.

In the control group (n=20), an identical incision was made through the body wall and the nerve cord was exposed. However, in this group, the ventral nerve cord was not severed. After transection and between testing, worms in all groups were kept individually in 6 cm diameter petri dishes filled with 20 ml of dechlorinated water.

Behavioral testing

Behavioral testing of swimming and reversal responses in individual worms was done before and at varying intervals after transection. A total of 20 transected worms were tested and each worm was stimulated three times. For testing, worms were individually placed in a 10 cm diameter glass finger bowl filled with 200 ml of fresh water. This allowed sufficient depth and space for the worm to execute locomotor responses to touch. Touch stimuli were delivered with a hand-held flexible probe that was designed to minimize potential injury to worms. The probe consisted of a thin rubber band loop attached to a wooden applicator stick. The diameter of the rubber band was 0.5 mm and the width and length of the loop were both approximately 1.5 cm.

In normal worms, two alternative and stereotyped locomotor responses are evoked in “open water” conditions in response to tail or head touch; these are the swimming and reversal responses, respectively (Drewes, 1999). To evoke the swimming response, the rubber loop was held above the worm at an angle of approximately 45°. The worm was then touched briefly and briskly by pressing the loop onto the posterior end of the worm. In normal worms, the response to tail touch consists of a series of rhythmic, undulatory

swimming movements. These movements consist of helical waves that move posteriorly along the body and propel the worm forward. Wave frequency is approximately 10 Hz and a typical swim episode lasts less than 1 s (Drewes, 1999).

To evoke the reversal response, the anterior end of a worm was touched with the rubber loop. In normal worms, the initial response to such stimuli involves rapid coiling of the posterior end immediately followed by an anteriorly directed wave of uncoiling. The result is an approximately 180° reversal of the body that is completed, as a stereotyped response, in about 0.5 sec (Drewes, 1999)

Behavioral responses of normal and regenerating worms were recorded on videotape using a JVC model TK 1070 color video camera and model HRS 7300U videocassette recorder. The camera was mounted on an Olympus Model SZ CTV dissecting microscope. Frame-by-frame playback of video recordings was used to determine the qualitative nature and axial localization of behavioral events (i.e., swimming or reversal).

Electrophysiological testing

Giant nerve fibers in *Lumbriculus* and other oligochaetes are known to mediate rapid escape shortening in response to tactile stimulation (Drewes, 1984; Zoran & Drewes, 1987). To test for functional recovery of action potential conduction in giant nerve fibers, worms were transferred individually to a moistened, printed-circuit-board grid of electrodes (Fig. 1). Under these conditions, the worms' repertoire of reflexive responses to touch includes rapid escape shortening and slows peristaltic crawling in forward and rearward directions. Swimming and reversal responses are not seen, since initiation of these behaviors requires a context of "open water" conditions.

Conduction of giant nerve fibers was studied using non-invasive electrophysiological recording procedures previously described by Drewes & Fournier (1990) and Rogge & Drewes (1993). To determine a timetable of functional recovery from ventral nerve cord transection, repeated testing was done in each worm before transection and at 3, 5, 10 and 24 hours after transection. A total of 34 worms were tested.

All-or-none giant nerve fiber spikes were evoked by very light tactile stimulation delivered with a hand-held probe. The tip of the probe consisted of a straight rubber band (1 cm long, 0.5 mm diameter) attached to the end of wooden applicator stick. A light touch of anterior segments evoked one or a few MGF spikes that were conducted posteriorly. A light touch of posterior segments evoked one or a few LGF spikes that were conducted anteriorly. Spikes were readily categorized as originating from either the MGF or LGF based on previously established criteria which include stereotyped differences in directionality, polarity, and spike amplitude (cf., Drewes & Fournier, 1989; Drewes & Fournier, 1990).

When re-testing worms after transection, the original surgery/transection site was readily recognized because two to three segments (< 1 mm) at this site remained slightly "swollen" (due to segmental shortening) compared to adjacent segments. The worm was aligned so that this "swollen" site was midway between the two pairs of recording electrodes. The center-to-center distance between the two electrode pairs was 6 mm. Cursors on a digital storage oscilloscope (Tektronix Model TDS 210) were then used to determine the peak-to-peak conduction time for giant fiber spikes across this 6 mm inter-electrode conduction path at various times before and after transection. For the electrophysiological measurements, conduction times after surgery were compared to before values using a paired-difference t test.

Histology

Additional sets of worms were designated for histological study and subjected to identical transection procedures. Worms were divided into four groups, consisting of ten worms each. Each group was sacrificed after a recovery time that corresponded to the same timetable for neurophysiological and behavioral testing (i.e., 0, 3, 10 and 30 h after transection).

Prior to fixation, worms were immobilized with 0.25 mM nicotine for 15 min and pinned to a silicone rubber dish. They were then immersed overnight in 2.5 % glutaraldehyde in 0.1 M cacodylate buffer at 5 °C. After fixation, a five-segment-long fragment of the worm was excised. The center of this fragment included the surgery/transection site. Fragments were washed in a 0.1 M cacodylate buffer and immersed in 1% OsO₄ in 0.1 M cacodylate buffer for 2 h. After washing fragments in double-distilled water, they were dehydrated in a graded series of ethanol. Then tissues were cleared, infiltrated in acetone, and embedded in Epon-Araldite at 60 °C. Sections (1 μM thick) were stained with 0.1% toluidine blue and observed with a camera-mounted compound microscope.

Results

Normal behavioral responses

In normal worms, touch-sensory fields for swimming (S) and reversal (R) are complementary and opposed, as shown by the sensory field maps in Fig. 2 (Normal). Light touch to any segments within the anterior half of the body induced a stereotyped reversal behavior (see Methods). Light touch to any segments within the posterior half of the body

invariably induced helical swimming. In all worms there was a slight overlap zone (equal to approximately 5-10% of a worm's body length) for the two sensory fields. When touched in the overlap zone, the worm responded by either reversing or swimming, or infrequently both.

Four points (hereafter designated P1-P4) along the body of the worms were preselected for repeated testing of the touch-evoked behavior responses (Fig. 2). P1, an anterior point close to the head end, was exclusively within the sensory field for reversal. Before surgery, in normal and experimental worms, all worms responded to touch stimuli at P1 by reversal of the whole-body. The second selected point, P2, was in the original sensory field for swimming. This point was several segments anterior to the transection site. The third and fourth points, P3 and P4, were also in the sensory field for swimming. However, P3 was several segments posterior to the transection site while P4 was close to the tail tip.

Before transection, each worm was tested to confirm whether swimming or reversal was evoked in response to touch stimuli at points P1-P4 (three trials per point). At this time, results were identical in all worms. Reversal responses were always evoked by stimulation at P1, whereas, stimulation at sites P2-P4 always induced helical swimming responses.

Post-transection responses to touch at P1

At 3 h after transection, 20 of 20 worms (100%) responded to touch at P1 by exhibiting an incomplete, rather than whole-body, reversal response. Such reversal, which was initiated in segments just anterior to the transection site, resulted in a rapid coiling that was clearly restricted to this anterior region and did not include segments posterior to the transection site. This coiling was immediately followed by an anteriorly directed wave of uncoiling. Since segments posterior to the transection site were not actively involved in the response, the result was an incomplete reversal.

At 8 h after transection, 10 of 20 worms (50%) showed no change from the response pattern seen at 3 h. On the other hand, the other 10 worms (50%) showed clear-cut indications of functional recovery. That is, when these worms were touched at P1, whole-body reversal responses were executed and these appeared indistinguishable from normal reversal responses. At 12 h after transection, all 20 worms exhibited whole-body reversal responses when touched at P1.

Post-transection responses to touch at P2

At 3 h after transection, 20 of 20 worms (100%) responded to touch at P2 by exhibiting incomplete, rather than whole-body swimming responses. Such swimming was initiated in head segments, as in normal worms, but helical undulatory movements of the body did not progress posteriorly beyond the transection site. The videotape recordings confirmed that segments posterior to the transection site were passively moved by active waves generated in segments anterior to the transection site.

At 8 h after transection, 11 of 20 worms (55%) showed no change in the response patterns seen at 3 h. On the contrary, 9 of 20 worms (45%) showed functional recovery at this time. In these worms, touch at P2 evoked helical swimming movements that were initiated in head segments and actively progressed into segments posterior to the transection site. At 12 h after transection, all 20 worms (100%) exhibited whole-body swimming responses when P2 was touched.

Post-transection responses to touch at P3

At 3 h after transection, 20 of 20 worms (100%) responded to touch at P3 by exhibiting incomplete reversal responses. Such responses were initiated at the tail end, and involved rapid coiling and uncoiling of segments posterior to the transection site. Reversal

of the whole-body was not completed because segments anterior to the transection site did not respond to touch at P3 and thus mechanically impeded reversal of posterior segments.

At 8 h after transection, 10 of 20 worms (50%) showed no change from behavioral response patterns seen at 3 h. However, the other 10 of 20 worms (50%) showed whole-body swimming responses when P3 was touched. These swimming responses were initiated at the head end of the worm and helical waves actively progressed past the transection site into posterior segments. At 12 h after transection, all 20 worms exhibited whole-body swimming when P3 was touched.

Post transection responses to touch at P4

At 3 h after transection, 20 of 20 worms (100%) responded to touch at P4 by exhibiting incomplete swimming responses. Such responses were initiated in segments immediately posterior to transection site. These waves progressed posteriorly toward the tail but no active swimming movements were seen in segments anterior to the transection site.

At 8 h after transection, 11 of 20 worms (55%) showed identical response pattern to worms tested at 3 h. However, nine of 20 worms exhibited normal, whole-body swimming responses with helical waves that were initiated at the head end and actively progressed posteriorly past the transection site. At 12 h after transection, all 20 worms responded to touch at P4 with normal, whole-body swimming.

For comparison, we tested swimming and reversal behavior in worms that had been amputated at a site that corresponded to the site of ventral nerve cord transection. The results indicated that there were immediate changes in behavioral response patterns for both fragments. The anterior fragment responded to touch stimuli at each of its ends in a whole-body fashion. That is, the fragment reversed when touched at its anterior end and initiated

swimming movements when touched at its posterior cut end. Thus, the response patterns appeared identical to those initially seen in segments anterior to ventral nerve cord transection (Fig. 2). The posterior fragment also responded to touch stimuli in a whole-body fashion. The fragment swam when touched at its posterior end and it initiated body reversal movements when touched at its anterior cut end. These response patterns were identical to those initially seen in segments posterior to ventral nerve cord transection (Fig. 2).

Electrophysiological testing

Figure 3A shows electrophysiological recordings of MGF and LGF spikes in response to head and tail touch, respectively. The normal time for MGF spike conduction across the 6 mm distance between adjacent electrode pairs was approximately 0.6 ms (Fig. 3A; Fig. 5). This corresponded to a velocity of 10 m/s. Normal time for LGF spikes conduction across the same distance was approximately 1.0 ms, corresponding to a velocity of 6 m/s (Fig. 3A; Fig. 5).

At 3 h after ventral nerve cord transection, touch-evoked MGF and LGF spikes were conducted up to, but not across, the transection site (Fig. 3B). However, at the next time of testing (10 h), through-conduction of giant fiber spikes (i.e., 1:1 coupling of spikes across the transection site) was re-established in some worms, as indicated by the presence of MGF and/or LGF spikes recorded by both pairs of electrodes (Fig. 3C).

Figure 4 shows the percentage of worms with recovery of MGF and LGF spike conduction across the transection site at various times during recovery. At 10 h, 32% of worms showed restoration of MGF through-conduction, while 15% of worms showed restoration of LGF through-conduction. At 20 h, the majority of worms (80% and 70%) showed through-conduction of both MGF and LGF spikes, respectively. With further

recovery, these percentages continued to increase until 30 h, when all worms showed re-establishment of both MGF and LGF through-conduction.

To track time-dependent changes in conduction, we selected a subset of five worms, all of which showed behavioral and electrophysiological signs of giant fiber regeneration by 10 h after transection (Fig. 5). At this time, the average MGF conduction time across a 6 mm distance that included the transection site, was 1.27 ± 0.06 ms ($n=5$), or about twice that in normal worms (Fig. 5). The average LGF conduction time over the same distance was 1.54 ± 0.3 ms. By 20 h the MGF conduction time decreased to 0.75 ± 0.03 ms, and the LGF conduction time decreased to 1.44 ± 0.1 ms. At 30 h, the MGF conduction time had decreased to a nearly normal value (approximately the same as before transection). In contrast, the LGF conduction time decreased more slowly and had returned to an approximately normal value by 130 h after transection.

Histology

Figure 7 shows longitudinal sections through the transection site at 3, 10, and 30 h after transection. At 3 h, serial sections failed to reveal any detectable cellular continuity between the severed giant fibers. There appeared to be a gap separating the two cut ends of ventral nerve cord. This gap was not filled by neuropil or giant fiber-like tissue. At 10 h, we were able to clearly trace neuropil-like tissue across the entire transection region. Examination of serial longitudinal sections revealed small-caliber outgrowths of giant nerve fibers that bridged the transection site. By 30 h, the giant fiber outgrowths appeared to be larger in diameter than at 10 h and neuropil-like tissues more extensively infiltrated the transection site.

Discussion

A key finding in this study is that nicotine can be used as a reliable paralytic agent that completely immobilizes worms, allows surgical access to the ventral nerve cord, and blocks segmental autotomy responses during and after surgery. During immersion in nicotine (0.25 mM for 15 min), touch-evoked locomotor responses (i.e. body reversal, swimming and crawling) were temporarily but completely blocked. In control worms, such nicotine effects were reversible and locomotor response patterns appeared normal within 3 h of recovery. A similar pattern of recovery from nicotine effects occurred in worms with transected ventral nerve cords. Subsequent regeneration of reflex pathways in the ventral nerve cord occurred at about 8-10 h after transection, which was well after nicotine effects had subsided. Consequently, we presume that the progress and outcome of regeneration was unaffected by nicotine, although we can not completely discount the possibility that brief exposure to nicotine somehow altered the timing of functional recovery of pathways within ventral nerve cord.

After the effects of nicotine subsided, there were immediate signs of reorganization of locomotor response patterns in worms with a transected ventral nerve cord. As a result, each portion of the worm was independently capable of performing swimming and reversal responses much like those in an intact worm. Comparable changes in response patterns were seen in amputated body fragments. Thus, transection of the ventral nerve cord, with or without disconnection of the surrounding body wall, triggers immediate alterations in reflex performance of each body portion.

Such rapid reorganization of locomotor responses has obvious adaptive significance. Because *Lumbriculus* has the ability to readily autotomize and slowly regenerate missing

body parts (Lesiuk & Drewes, 1999), it seems important that surviving fragments immediately reorganize reflex responses in order to continuously provide reflex capabilities for predator avoidance. The reorganization of locomotor responses in *Lumbriculus*, after ventral nerve cord transection, or segment amputation, demonstrates the remarkable functional plasticity of this worm's central nervous system. Mechanisms for achieving such plasticity are unknown.

Our experiments involving ventral nerve cord transection created an "unnatural" situation in which the body wall surrounding the transection site was not completely severed. Thus, worms were not physically separated. This allowed the two cut ends of ventral nerve cord to remain in close proximity and reconnect by regenerative processes. By repeatedly testing behavioral and electrophysiological performance of worms, we were able to establish, for the first time in any aquatic oligochaete, a timetable for regeneration of the ventral nerve cord.

The first indication that ventral nerve cord regeneration had occurred was the re-establishment of the whole-body swimming and reversal responses. This happened as early as 8 h after transection. In order to produce whole-body swimming in response to tail touch, two types of central reconnections must necessarily occur. First, the ascending central pathway, which conducts or relays information from posterior touch-sensory pathways to anterior circuits that initiate swimming, must regenerate across the transection site. Second, the descending intersegmental pathways (presumably a central pattern generator) that mediate swimming must also regenerate. Similarly, for the worm to produce whole-body reversal, there must be reconnection of a descending central pathway that conducts or relays information from anterior touch-sensory pathways to posterior circuits where initiation of

body reversal (i.e. coiling) occurs. Finally, the ascending pathway crucial for executing later stages of reversal behavior (i.e. waves of uncoiling) must also regenerate. It has been previously suggested, on the basis of the relatively slow velocity of the moving waves, that the pathways responsible for swimming and reversal are probably not mediated by giant nerve fibers (Drewes, 1999). Our results clearly indicate that functionally intact giant fiber pathways are not necessary for successful execution of whole-body swimming and reversal responses, since restoration of these responses occurred earlier than restoration of through-conduction in the giant fibers.

Precise determination of the onset of functional restoration in reflex pathways that mediate whole-body locomotor responses yield insights and confirmation as to the independence of the central pathways that mediate these reflexes. For example, if we look at the regeneration timetable for a typical worm, we see that the swimming response was restored at 10 h after transection, while the reversal response was restored at 12 h. This was followed by restoration of MGF and LGF through-conduction at 18 h. Not all worms showed such clear temporal separation in recovery, presumably because testing was done at intervals of several hours and therefore the precise moment for recovery of each pathway was missed. However, the fact that at least some worms showed such temporal separation in recovery is strong evidence for the independence of the central pathways mediating these responses.

Functional restoration of through-conduction in giant nerve fibers was studied using non-invasive electrophysiological techniques. Recordings of MGF and LGF spikes were made with two pairs of electrodes straddling the transection site (Fig. 1; Fig. 3). Such recordings permitted unequivocal identification of success or failure of giant fiber through-

conduction. In some worms, restoration of through-conduction appeared as early as 10 h after transection, which is still somewhat longer than recovery of swimming and reversal responses (8 h).

The time required for *Lumbriculus* to reestablish through-conduction of MGF and LGF spikes across the transection point is much shorter than any regeneration times previously reported in earthworm giant fibers, or interneurons in any other invertebrates (for review see Drewes et al., 1988). The rapidity in regeneration, compared to earthworm, may be due to several factors. First, the physical separation, after ventral nerve cord transection, in *Lumbriculus* was estimated to be only about 0.25 mm. This is much smaller than the separation seen after ventral nerve cord transection in larger earthworm species. Second, segmental regeneration is known to be much more robust in *Lumbriculus* than in any earthworm. Perhaps the rapid repair and reconnection of giant nerve fibers is a specific reflection of this worm's general capacity for segmental regeneration.

Giant fiber regeneration in earthworms, following ventral nerve cord transection, occurs by outgrowth and cell-specific reconnection of sprouts arising from the cut ends of giant fibers (Drewes et al., 1988). We presume that the giant fiber regeneration in *Lumbriculus* occurred by the same sprouting process, only faster. Initial onset of restored through-conduction of giant nerve fibers was accompanied by abnormally long conduction time. Gradually, from 10-20 h after transection, there was a substantial reduction in these times across the transection site. Conduction time continued to decline slowly thereafter, eventually reaching normal values, presumably due to a progressive increase in the diameter of the outgrowth that join the giant fibers. Presence of small-caliber outgrowths from giant fibers may be the basis of the relatively long conduction time. At a later time, larger

diameter connections were noted (Fig. 7) and conduction times through the transection site were reduced.

One possible reason why non-giant pathways reconnect more quickly than giant fiber pathways may be due to the size-mismatch between the giant nerve fibers and their interconnecting sprouts. Even though reconnection of all fibers may occur at about the same time, the initial size mismatch between small sprouts and the giant fibers from which they arise may prevent through-conduction. On the other hand, less size-mismatch between non-giant pathways and their sprouts may enable earlier through-conduction across the transection site.

In this study, we clearly showed that *Lumbriculus* is a robust model for studying neural plasticity and ventral nerve cord regeneration. Our procedures allow surgical invasion of the ventral nerve cord and provide several different indicators of functional and behavioral recovery during regeneration. This raises the possibility of studying regeneration within the context of more complicated and unusual surgical paradigms, such as ventral nerve cord ablation.

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Figure Legends

Fig. 1. Diagram of non-invasive electrophysiological recording set up. Worms were placed on the printed-circuit board along side a plexiglass strip. There are two recording channels (Ch1, Ch2) that detect action potential from posterior sites of the body on either side of the ventral nerve cord transection site. Touch stimuli were delivered with a hand-held probe used to evoke giant fiber spikes.

Fig. 2. Maps of touch-sensory fields for swimming and reversal in normal worms and worms with transected ventral nerve cords. In a normal worm (left) P1-P4 indicate points where tactile stimuli were delivered to test behavioral responses. The intended transection site (arrow) was between P2 and P3. Bracket R indicates sensory field for reversal and bracket S indicates sensory field for swimming. The percentage of tests which evoked swimming or reversal are shown for the four stimulus sites before and at three times after transection. Swimming and reversal responses that were restricted to segments anterior to the transection are indicated with Ra and Sa. Swimming and reversal responses that were restricted to segments posterior to the transection are indicated with Rp and Sp. Responses involving whole-body swimming are indicated with S and whole-body reversal are indicated with R.

Fig. 3. Simultaneous recordings of MGF and LGF spikes from two recording electrodes before and after transection. A) Before transection, MGF and LGF spikes were reliably conducted across a 6 mm distance between the two recording channels. The intended transection site is labeled with arrow. B) At 3 h after transection, an anteriorly conducting LGF spike failed to conduct across transection site. Likewise, a posteriorly conducting MGF spike also failed to conduct across transection site. C)

At 20 h after transection, both MGF and LGF spikes were successfully conducted through the transection site, although conduction times were longer. Voltage scale = 100 μ V. Time scale = 1 ms.

Fig. 4. Graph showing percentage of worms with successful MGF and LGF through-conduction at 0, 3, 10, 20, and 30 h after transection (n=34).

Fig. 5. MGF and LGF conduction times across the transection site at 0, 10, 20, 30, 45, 72 and 130 h of a group of worms that showed through-conduction at 10 h. The conduction times were measured over 6 mm distance, which included the transection site. Each bar indicates the mean conduction time \pm SEM, n=5.

Fig. 6. Frontal longitudinal sections of *Lumbriculus* transected segment (arrow), before, 3, and 20 h after transection. **A)** In a normal (or before transection) worm, the medial and two lateral giant fibers (asterisk). **B)** At 3 h after transection, giant fibers were separated at the transected site. **C)** At 20 h after transection, All 3 giant nerve fibers were seen in the transected segment. Scale: 20 μ m.

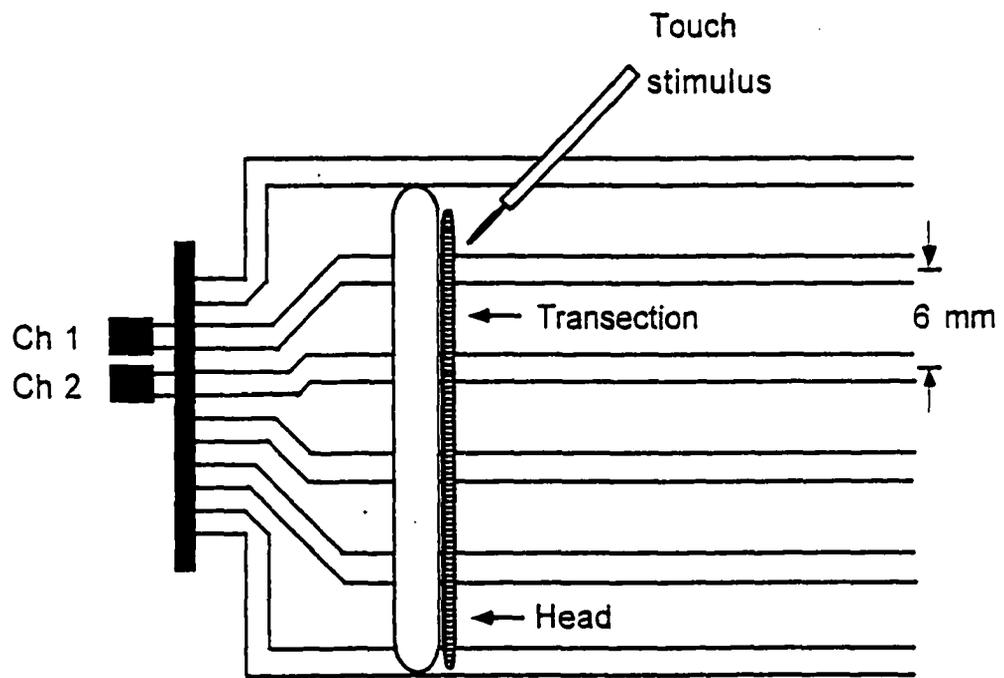


Figure 1.

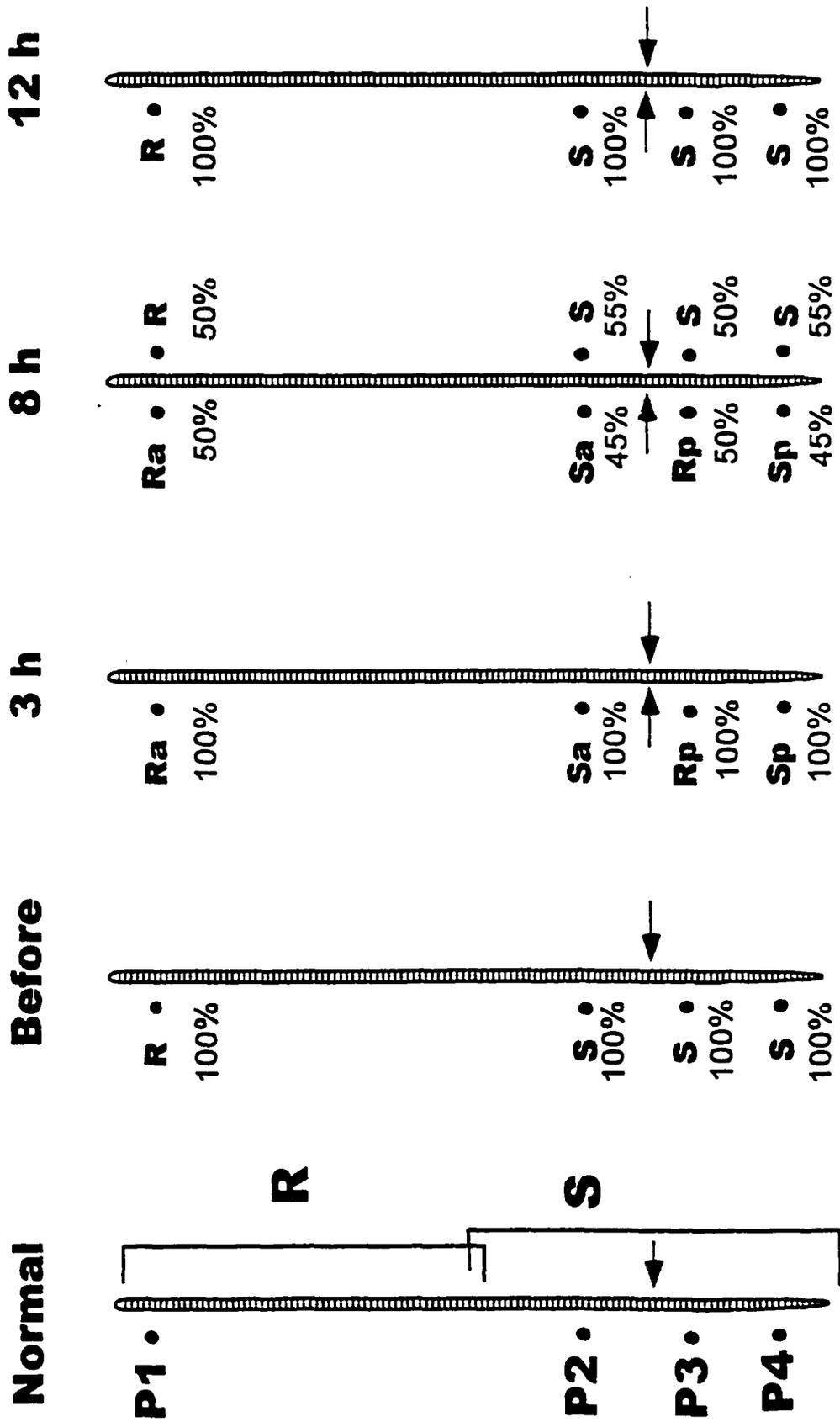


Figure 2.

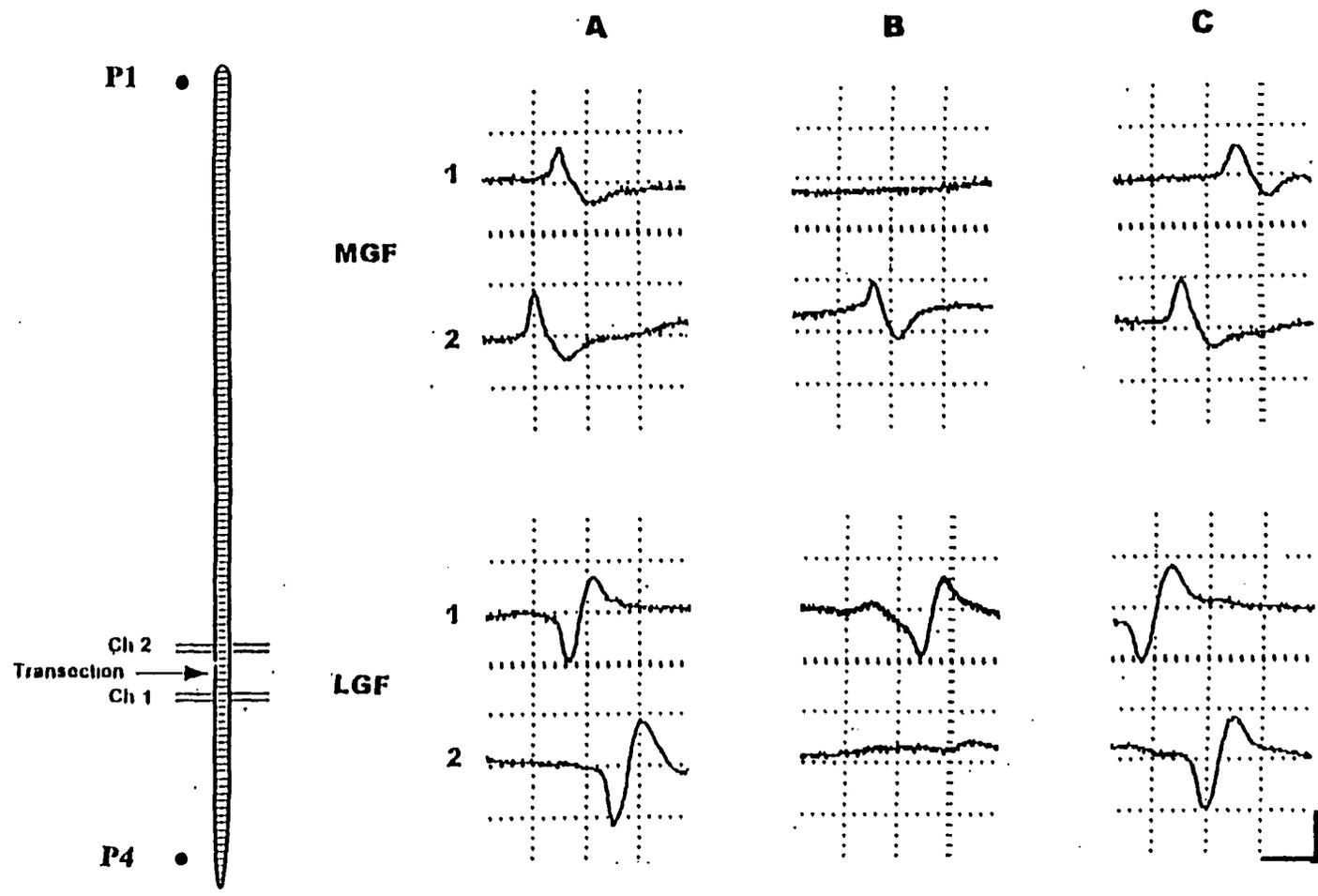


Figure 3.

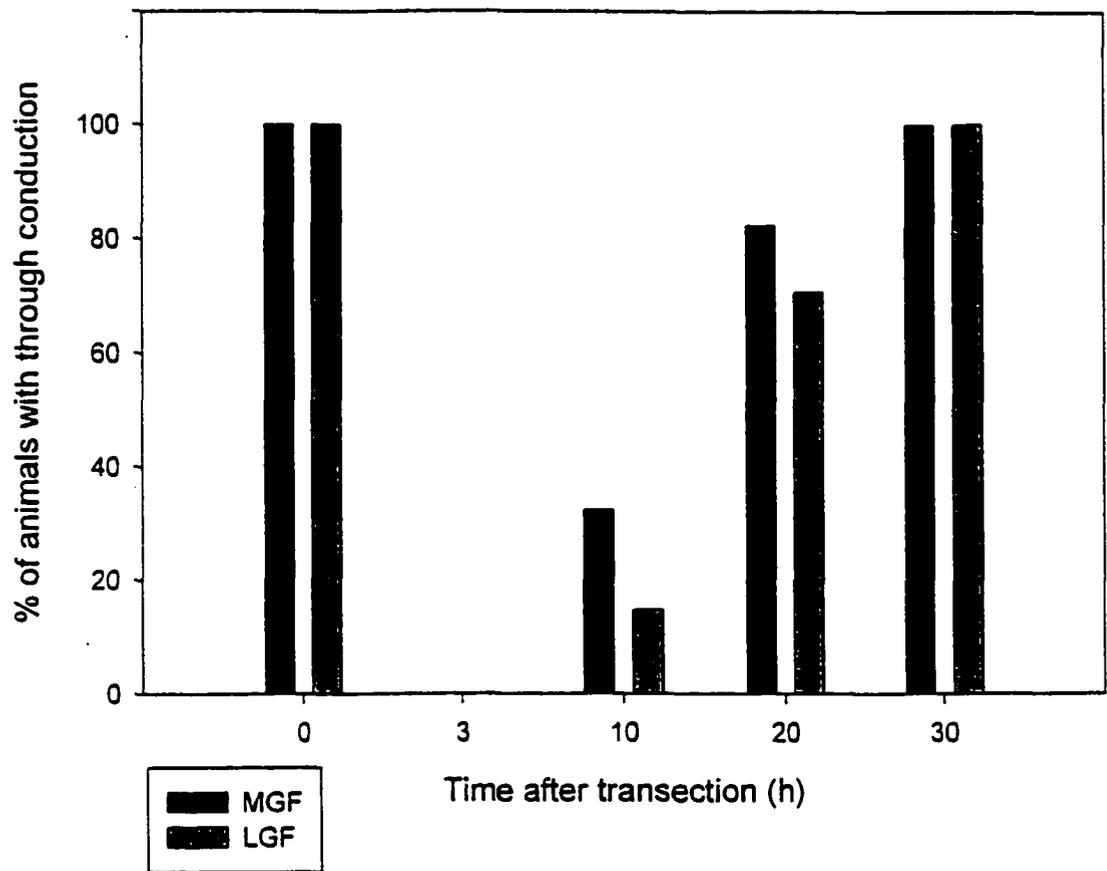


Figure 4.

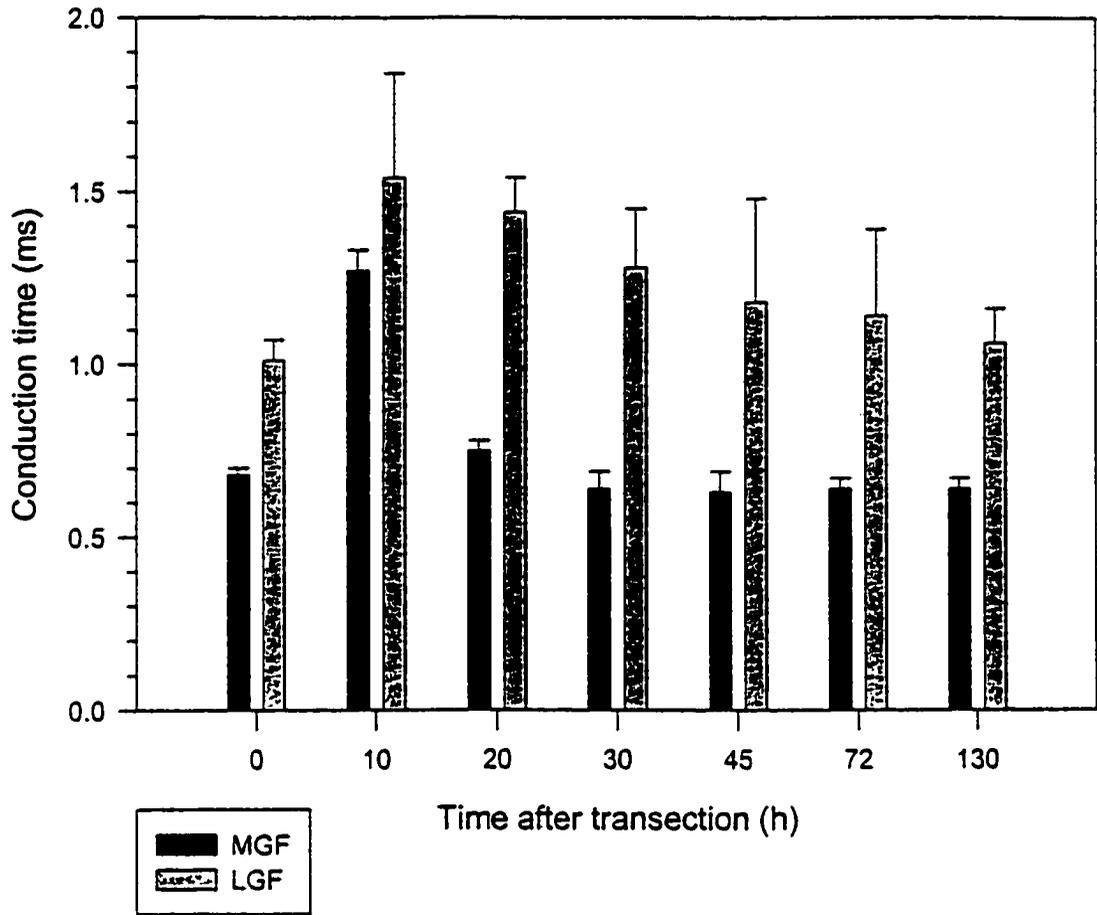


Figure 5.



Figure 6.

CHAPTER 4. BEHAVIORAL PLASTICITY AND CENTRAL REGENERATION OF LOCOMOTOR REFLEXES IN THE FRESHWATER OLIGOCHAETE, *Lumbriculus variegatus*. II: ABLATION STUDIES

A paper to be submitted to Journal of Invertebrate Biology

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Abstract

Eight to ten segments of ventral nerve cord were surgically ablated from a posterior location in *Lumbriculus variegatus*. Immediately after surgery, touch-evoked locomotor responses could be initiated both in segments anterior and posterior to the ablation site; however, responses in these two regions were independent and uncoupled. As recovery progressed, four types of outcomes at the ablation site were observed: (a) recovery of normal functions with no outgrowth, (b) formation of an ectopic head, (c) irregular and abnormal outgrowths, and (d) segmental autotomy. Further studies were carried out in the first two groups to more fully characterize touch-evoked swimming and body reversal behaviors and to electrophysiologically assess sensory fields and conduction properties of giant nerve fibers at or near the ablation site. In some of the worms in group 1, there were clear-cut behavioral and electrophysiological signs of recovery and reconnection by 3 d after ablation. By 8 d, all worms had recovered and exhibited response patterns comparable to those of normal worms. Group 2 worms, with an ectopic head, showed persisting alterations of locomotor reflex responses, as seen immediately after ablation. Thus, segments posterior to the ablation, in combination with segments in the ectopic head, exhibited touch-evoked swimming and body reversal responses resembling those of a complete worm. These responses occurred independently of touch-evoked locomotor responses in segments anterior to the ablation

which were independently capable of similar locomotor responses. Sensory fields for medial and lateral giant nerve fibers were also altered in worms with ectopic heads, again reflecting a configuration expected for two worms. Specifically, through apparent morphallactic reorganization, there was emergence of medial giant fiber sensory field that included the ectopic head and 10-15 segments posterior to the ablation site. In contrast, electrophysiological recordings indicated that longitudinal through-conduction of giant nerve fiber spikes, across the ablation site, was re-established, thus electrophysiologically linking the two portions. Histological examination revealed that the giant nerve fibers in the ectopic head were connected to the main body axis. These results provide a new experimental paradigm and new insights into the phenomenon of central neural plasticity and morphallactic reorganization in oligochaetes.

Additional keywords: giant nerve fibers, locomotion, reflexes, ectopic head, neural plasticity

Introduction

It is well established that giant nerve fiber pathways (interneuronal in origin) in the ventral nerve cord of oligochaete worms are capable of rapid and reliable reconnection after ventral nerve cord transection (Drewes et al., 1988). Previous studies of such reconnection have focused on terrestrial oligochaetes, such as *Lumbricus terrestris* and *Eisenia foetida* (Balter et al., 1980; Vining & Drewes, 1985). In these species, the timing of functional recovery (i.e., restoration of giant nerve fiber through-conduction after transection) varies from about 1-2 d in *Eisenia* (small redworm) to 5-18 d in *Lumbricus* (large nightcrawler). In the preceding study (Lesiuk & Drewes, 2000), functional recovery of giant nerve fiber

through-conduction after transection was demonstrated in an aquatic counterpart of earthworms, *Lumbriculus variegatus* (common name: blackworm). The results showed exceptionally rapid reconnection of central pathways after ventral nerve cord transection. Functional restoration of the non-giant nerve fiber pathways that control whole body locomotor behaviors, such as swimming and reversal, occurred as early as 8 h after transection. Restoration of giant nerve fiber through-conduction occurred slightly later, often about 10 h after transection (Lesiuk & Drewes, 2000).

In the present study, we examine plasticity and repair of central pathways in *Lumbriculus* within a more challenging context than transection - namely, complete ablation of 5-8 segments of ventral nerve cord. Several divergent outcomes were observed. First, in some worms, functional restoration of both giant and non-giant pathways was observed after ablation. A second outcome, in other worms, was rapid formation of a lateral ectopic head at the ablation site. This unexpected finding provided an opportunity to (1) study how neural elements in the ectopic head interface with and influence the reconnection of central neural pathways in the main body axis and (2) determine how pre-existing patterns of reflex organization in segments near the ablation site, are influenced by the presence of an ectopic head. The technical ability to induce formation of an ectopic head thus provides new insights in regard to neural plasticity and segmental pattern formation in oligochaetes.

Materials and Method

Animals

Lumbriculus variegatus (Order Lumbriculida, Family Lumbriculidae) were obtained from established, asexually reproducing laboratory cultures. The substrate consisted of

brown paper towel submerged in aged tap water. Worms were kept at room temperature (23°C) and fed twice weekly with sinking fish food pellets. Only worms that showed longitudinal uniformity in segmental size and pigmentation were used in experiments.

In all ablation experiments, worms were immobilized by immersion in 20 ml of 0.25 mM nicotine for 15 min. After this exposure, the worms exhibited neither reflex response to touch stimuli nor spontaneous muscle movements. They were relaxed and body length was slightly longer than under normal conditions. Pulsations of dorsal and lateral blood vessels were still apparent.

After immobilization, worms were removed from nicotine, rinsed briefly in double-distilled water and transferred to a silicone rubber dish containing spring water. Minutien insect pins (stainless steel; 0.05 mm diameter) were then used to pin worms lateral side up to the silicone rubber dish. The two pins were positioned approximately 12 body segments apart. A narrow strip of the ventral body wall, along with the underlying ventral nerve cord for a distance of five to eight segments between the pins, was removed using microdissecting scissors (Vannas style iris spring scissors, 3 mm cutting edge). Precautions were made to avoid injuring the ventral blood vessel and intestine. No sutures or other post-surgical treatments were used to enhance recovery. After surgery, worms were transferred to individual containers (5 cm diameter plastic petri dishes filled with 10 ml of dechlorinated water) for recovery and regeneration.

Behavioral testing

Swimming and reversal responses were tested at three different times in each worm: before surgery and at 3 days and 8 days after ablation. Each worm was tested in a 10 cm diameter glass finger bowl containing 200 ml of spring water. A hand-held stimulus probe

was used to evoke locomotor reflex responses under these “open water” conditions. The probe was made from a thin rubber band loop (diameter 0.5 mm) attached to a wooden applicator stick. The diameter of the rubber loop was approximately 1.5 cm (Drewes, 1999).

In normal worms, touching the body with the loop evokes one of two stereotyped locomotor responses. The first behavioral response, helical swimming, is evoked by touching posterior segments. The swimming consists of a brief, rhythmic series of helical undulations of the body. As these helical waves progress posteriorly along the body, they propel the worm forward. In *Lumbriculus*, the frequency of helical waves is approximately 10 Hz and a swim episode lasts about 1 s (Drewes, 1999).

The second behavioral response to touch is body reversal. This is evoked by touch to anterior segments. During reversal, the worm rapidly coils its posterior end and this is followed by an anteriorly directed wave of uncoiling. The result is an approximately 180° reversal of the body which occurs in about 0.5 s (Drewes, 1999).

Behavioral responses were recorded on videotape using a JVC model TK 1070 color video camera and model HRS 7300U videocassette recorder. The camera was mounted on an Olympus Model SZ CTV dissecting microscope. Video recordings were used to determine (a) which type of locomotor response (i.e., swimming or reversal) was evoked by a stimulus, and (b) the localization or generalization of these locomotor movements along the body axis.

Electrophysiological testing

Impulse conduction of regenerating giant nerve fibers was studied using non-invasive electrophysiological recordings (Drewes & Fournier, 1990; Rogge & Drewes, 1993). Each worm was rinsed briefly in double-distilled water and transferred to a moistened, printed-

circuit-board grid of electrodes. Excess water was removed using a Kimwipe. While on the circuit board, touch stimuli were delivered by a hand-held flexible probe (a half inch long straight rubber band attached to an applicator stick). Worms may respond to such stimuli by initiating all-or-none spikes in giant nerve fibers. Medial giant fiber (MGF) and lateral giant fiber (LGF) can be unequivocally distinguished from one another based on differences in directionality, polarity and spike amplitude (Drewes & Fournier, 1989; Drewes & Fournier, 1990). When multiple spikes are evoked, worms produce rapid escape shortening of the head or tail end.

Signals from recording electrodes were pre-amplified using a pair of differential recording amplifiers (100x gain, capacity-coupled inputs). Signals were displayed on a digital storage oscilloscope (Tektronix model TDS 210). At various times before and after ablation, giant fiber conduction properties were assessed. MGF and LGF conduction times were measured over a 6 mm inter-electrode conduction path, as indicated by the peak-to-peak interval between corresponding giant fiber spikes in the two recording channels. Three replicate measurements were taken for the MGF and LGF during each test period (before ablation, 3 days and 8 days after ablation). Conduction times after ablation were compared to those before using a paired difference t-test.

Histology

At 8 d after ablation, worms were placed in a spring water overnight to clear the intestinal contents. Prior to fixation, nicotine (0.25 mM) was used to immobilize the worms. After immobilization, worms were pinned to a silicone rubber dish and immersed overnight in 2.5 % glutaraldehyde in 0.1 M cacodylate buffer at 5 °C. After fixation, a ten-segment-long fragment of the worm was excised. The center of this fragment included the ablation

site and newly formed segments. Then, fragments were washed in a 0.1 M cacodylate buffer and immersed in 1% OsO₄ in 0.1 M cacodylate buffer for 2 h. They were rinsed in double-distilled water twice and dehydrated in a graded series of ethanol. After dehydration, tissues were cleared, infiltrated in acetone, and embedded in Epon-Araldite at 60 ° C. The tissues were cut into 1 μm sections and stained with toluidine blue for light microscopy.

Results

Outcomes from ventral nerve cord ablations in 120 worms were categorized into four different groups. In the first group, wound healing appeared normal and this was followed by full recovery, as described below. This result occurred in 47% of the worms; hereafter, this group will also be referred to as group 1 or "restoration group." A representative sample from this first group was used for subsequent behavioral and electrophysiological analysis.

In the second group, a head-like, multi-segmental, lateral outgrowth developed at the ablation site. This result occurred in 25% of the worms. Hereafter, these worms will be referred to as group 2 or "ectopic head formation" group. Some worms from this group were sacrificed for histological studies and therefore were unavailable for other testing. In the third group, representing 5% of all worms, abnormal outgrowths were found at the ablation site. Morphology of these outgrowths was variable. Some appeared as small amorphous and bulbous, lateral outgrowths. In some cases there were small, segmented and finger-like projections attached to the bulbous outgrowths. In rare cases, there were two or more outgrowths (head-like and/or tail-like in appearance) at the ablation site (Fig. 4). The outcome in the fourth group was segmental autotomy, which occurred within 24 h after

ablation. Autotomy occurred in 23% of the worms. Results here will focus on features of the first two groups described above.

Group 1: Restoration

Behavioral responses

In normal worms, the touch-sensory fields for swimming and reversal responses are configured as shown in Fig. 1 (before). Touch to segments in the anterior half of the worm invariably evokes a stereotyped reversal behavior in which the worm initiates a rapid coiling of the tail followed by an anteriorly directed wave of uncoiling. This results in approximately 180° reversal of the body. Touch to segments in the posterior half of the worm evokes helical swimming in which the worm initiates a rhythmic series of helical undulatory movements of the body starting at the head. Rearward progression of the waves propels the worm forward. There is approximately 5-10% of the body length of the worm that represents an overlap zone, within which touch elicits either reversal or swimming.

Four points along the body of the worms (designated P1-P4) were pre-selected for repeated testing of the behavioral responses to touch stimuli (Fig. 1). Before the ablation, all worms ($n = 14$) showed body reversal responses when P1 was touched and helical swimming responses when P2-P4 were touched. Thus, future ablation site, in between P2 and P3, was completely within the original sensory field for swimming.

At 1 d after ablation, all worms showed significant changes in behavioral responses (Fig. 1). When P1 was touched, the worms responded with a partial reversal behavior (Ra) involving only segments anterior to the ablation site. These segments exhibited a sequence of rapid coiling followed by a wave of uncoiling. When P2 was touched, the worms initiated

swimming movements (Sa) that were restricted to segments anterior to the ablation site; helical waves did not progress beyond the ablation site.

The most significant change occurred when P3 was touched, these worms responded with a "partial" reversal response (Rp) that was initiated at the tail end. The response consisted of a rapid coiling and uncoiling but these movements were restricted to segments posterior to the ablation site. When P4 was touched, the worms responded with partial swimming initiated at segments just posterior adjacent to the ablation site (Sp). The resulting helical waves progressed posteriorly toward the tail, but segments anterior to the ablation site were inactive.

At 3 d after ablation, 8 of 14 worms showed no change (in comparison to 1 d after ablation) in behavioral responses to touch at P1-P4. On the other hand, 6 of 14 worms showed significant changes in response patterns to touch at P1-P4 (Fig. 1). Patterns of responses in these five worms appeared restored and identical to those of normal worms. At 8 d after ablation, all 14 worms showed full restoration; behavioral responses to touch at P1-P4 were indistinguishable from those of normal worms.

Electrophysiological responses

The same 14 worms in this group were used for non-invasive electrophysiological testing. In normal worms prior to ablation, the MGF sensory field included only segments in the anterior 40% of the body length. The LGF sensory field included all segments in the posterior two thirds of the body. Just anterior to the middle of the body, MGF and LGF sensory fields overlapped in an area equal to about 5-10% of the worms' body length. When tested 8 d after ablation, the maps of MGF and LGF touch sensory fields in segments near the ablation site appeared unchanged in comparison to before ablation (Fig. 2).

Next, worms were tested for recovery of giant nerve fiber through-conduction across the ablation site. Worms were placed on the recording electrode grid with the ablation site in between two pairs of recording electrodes. The MGF and LGF spike conduction times (across the 6 mm interelectrode distance) were then measured and compared to conduction times prior to ablation. The latter times were 0.6 ± 0.007 ms and 1.02 ± 0.11 ms (mean \pm SEM), respectively. At 3 d after ablation, the MGF and LGF conduction times were 0.96 ± 0.2 ms and 1.59 ± 0.18 ms ($n=14$), and at 8 d after ablation, the times were 0.78 ± 0.08 ms and 1.07 ± 0.11 ms (Fig. 3). Such recordings indicated that, in each of the 14 worms, MGF and LGF through-conduction was re-established across the ablation site.

Group 2: Ectopic head formation

Behavioral responses

In this group an ectopic head projected laterally and perpendicularly to the longitudinal axis of the body. It consisted of a well-formed prostomium and five to six segments (Fig. 4A). The pigmentation in the ectopic head was much lighter than surrounding segments or segments in the original head. Ectopic head segments were motile and exhibited behaviors similar to those of a normal head end, such as searching movements and waves of peristaltic locomotion (i.e. crawling). From this group we selected 20 worms in which behavioral responses were tested by touching at P1-P4.

At 3 days after ablation, all 20 worms showed significantly altered but identical patterns of behavioral responses (Fig. 5). When P1 was touched, the worms responded with a partial reversal response (Ra) involving only segments anterior to the ablation. These segments initiated a rapid coiling followed by an anteriorly directed wave of uncoiling, but the ectopic head and segments posterior to it showed no active response to P1 stimulation.

When P2 was touched, worms responded with a partial swimming (Sa), in which helical swim waves occurred in only segments anterior to the ablation. The helical swim waves did not move posteriorly beyond the ablation site. When P3 was touched, worms exhibited localized reversal responses (Rp) that were similar to those in normal worms, except that movements were restricted to segments posterior to the ablation site. Finally, when P4 was touched, the worms responded with localized swimming movements (Sp) that were similar to those of normal worms, except that swimming movements were restricted to segments posterior to the ablation.

At 8 d after ablation, all worms in this group continued to exhibit the same behavioral response patterns seen at 3 d after ablation. The persistence of altered response patterns in this group contrast with the reversion of behavioral responses seen in group 1 (Figs. 1 & 5).

Electrophysiological responses

Worms with ectopic heads were placed on the recording electrode grid. Sensory fields were examined at 3 and 8 d after ablation. Results showed a change in the pattern of evoked giant fiber spiking when touched at P3, as compared to normal worms. In normal worms, touch at P3 invariably evokes an LGF spike. In contrast, in worms that had an ectopic head, touch at P3 evoked an MGF spike. This is a clear evidence of a switchover from LGF to MGF sensory fields (Fig. 7). To test the spatial limits of this emergent of MGF sensory field, we mapped sensory fields of segments within and near the ectopic head (Fig. 2). When any segment in the ectopic head was touched, an MGF spike was invariably evoked in the main body axis. In addition, the MGF sensory field included 1-2 segments anterior to the ectopic head as well as 10-15 segments posterior to the ectopic head. Sensory fields in the remaining segments were comparable to those of normal worms.

Next, worms were placed on the recording grid with the ectopic heads in between the two pairs of recording electrodes. Figure 6 shows electrophysiological recordings of MGF and LGF spikes in response to the head and tail touch before, at 3 d and 8 d after ablation. At 3 days after ablation, all worms with an ectopic head (n=20) showed no MGF or LGF spikes conduction across the original ablation site. However, touch-evoked MGF and LGF spikes were successfully conducted across this site at 8 days after ablation.

The time it took for MGF and LGF spikes to conduct between the two pairs of recording electrodes was measured while these electrodes straddled the ectopic head and original ablation site. At 3 d after ablation, such measurements were not possible since neither MGF nor LGF spikes were successfully conducted across the ablation site. However, at 8 d after ablation, successful conduction was seen in both MGF and LGF. The average conduction time for the MGF was 1.01 ± 0.08 ms and for the LGF 1.97 ± 0.75 ms. By comparison, in normal worms (before ablation), MGF and LGF conduction times in this region were 0.6 ± 0.01 ms and 1.17 ± 0.38 ms (mean \pm SEM), respectively (Fig. 8).

Histology

Frontal longitudinal sections of the ventral nerve cord were studied at 8 d after ablation in worms that had formed an ectopic head (Fig. 9). The courses and connection of the ventral nerve cord, especially the giant nerve fibers, was closely traced through serial sections. These sections revealed a clear-cut anastomosis of the ventral nerve cord in the ectopic head with the main body axis. The anastomosis appeared as a triangular rather than a simple T-like pattern (Fig. 9). The MGF was clearly present in the anastomosis branches, which join the ectopic head's ventral nerve cord to the main body axis.

Reconnections involving the LGF were more complex. In the cases of ectopic head formation on the right side of the body, the left LGF in the ectopic head appeared in serial section to be connected to the ipsilateral (right) LGF in segments anterior to the ablation. The right LGF in the ectopic head appeared connected to the ipsilateral (right) LGF in segments posterior to the ablation. Thus, there did not appear to be a continuous connection of the right LGF along the main ventral nerve cord axis, although such a continuous connection was clearly visible for the left LGF along the main ventral nerve cord.

Discussion

The ablation of five to eight segments of the ventral nerve cord in *Lumbriculus* represents a significant challenge in terms of nervous system repair. The ablation created a gap of at least 1-2 mm, or about 10-20 times greater than the diameter of the ventral nerve cord itself. In response to this challenge, two main outcomes were observed: no outgrowth (group 1) and ectopic head formation (group 2).

In all worms, there were immediate signs that the ablation procedure significantly disrupted the central neural pathways that mediate body reversal, swimming, and rapid escape. Lacking intersegmental continuity in the central nervous system, as a result of ablation, anterior and posterior portions of the worm became functionally disconnected and independently exhibited swimming and reversal responses (Fig. 1). These response patterns are comparable to those seen following simple nerve cord transection (Lesiuk & Drewes, 2000).

In one subset of worms with ablated nerve cords (group 1), reflex patterns eventually reverted back to normal (pre-surgical) configurations, indicating restoration of central

pathways that mediate these reflex responses (Fig. 1). These restorations were achieved by the reconnection of the two cut ends, presumably by the outgrowths from both ends of the ventral nerve cord. However, this reconnection differed from that seen in transection because it was slower and occurred over a substantially greater distance. It is not clear whether the tissue that bridged the two cut ends derived entirely from outgrowing axonal sprouts or whether there was *de novo* synthesis of new neurons that contributed to the bridge. Irrespective of the structural basis for reconnection, one functional outcome was restoration of giant fiber through-conduction across the ablation site. Another outcome was that MGF and LGF sensory field configurations remained as those of normal worms (Fig. 2).

These results contrast with those of group 2 in which there was formation of an ectopic head arising from the lateral body surface at the ablation site. By comparison, in a polychaete worm, *Ophryotrocha puerilis*, ablation of a portion of ventral nerve cord induced the formation of an ectopic tail from the ablation site (Pfannenstiel, 1984). A reasonable hypothesis to explain why different ectopic structures form in these two annelids is that, in one case (e.g., *Lumbriculus*) the anterior cut surface of the ventral nerve cord in segments posterior to the ablation site may be the primary inductive influence for ectopic head outgrowth. In the other case (e.g., *Ophryotrocha*) the posterior cut surface of the ventral nerve cord in the segments just anterior to the ablation site may be the primary inductive influence for ectopic tail outgrowth.

The nervous system in the ectopic head of *Lumbriculus* was merged with that of the main body axis, both anterior and posterior to the ablation site (Fig. 9). There were several important functional characteristics of this reconfigured ventral nerve cord. First, there was a persistence of the independent sensory fields for swimming and reversal responses in the

anterior and posterior portions of the worms (Fig. 5). This could be viewed as a failure to re-establish intersegmental pathways responsible for mediating whole-body reversal and swimming. Perhaps the ectopic head and its ventral nerve cord interfered with this reconnection by diverting outgrowths or suppressing reconnections arising from the severed nerve cord.

Second, there were interconnections of the giant nerve fibers in the ectopic head with the giant nerve fibers in the main body, but these occurred in a complex pattern (Fig. 9). Although cell-to-cell specificity appeared to be maintained in these connection (i.e., medial-to-medial and lateral-to-lateral giant nerve fibers), lateral specificity in these connections was not always maintained. For example, the left LGF in the ectopic head appeared connected to the right LGF in segments anterior to the ablation (Fig. 9). One functional consequence of these connections was re-establishment of MGF and LGF through-conduction in the ablation region (Fig. 6). Re-establishment of these connections contrasts with the apparent failure to reconnect pathways required for whole-body swimming and reversal responses. Thus, it appears that formation of reconnections between giant nerve fibers may not be subject to the same constraints as for reconnection of non-giant reflex pathways.

The third property of the nervous system in worms with ectopic heads was the emergence of a MGF sensory field, which included the entire ectopic head and numerous adjacent segments, especially posterior to the ablation site (Fig. 2). The emergence of the MGF field under these conditions is indicative of a morphallactic reorganization, comparable to which occurs during segmental regeneration in amputated worm fragments (Drewes & Fournier, 1990). We presume that factors mediating this reorganization emanate from the ectopic head and somehow project preferentially into segments posterior to the ablation site.

Furthermore, outgrowth of the new head may provide the cues and/or targets required for neural connections and pathways that support independent swimming and reversal capabilities in the posterior region of the worm (Fig. 5). In situations in which the ectopic head did not form (i.e., group 1) regeneration was perhaps so rapid that the signals from the posterior ventral nerve cord were insufficient to allow ectopic head induction.

Formation of an ectopic head and its accompanying features provide interesting comparisons with some other types of developmental studies. The first comparison is with oligochaete species, such as *Dero*, that undergo paratomy, in which *de novo* formation of a new head and tail segments occurs in tandem with other segments along the main body axis. The result is formation of anterior and posterior zooids that are structurally and functionally linked until fission (Drewes & Fournier, 1991). Prior to fission, neurophysiological and behavioral characteristics reflect those of a whole worm, rather than two separate worms. Though morphallactic reorganization of giant fiber sensory fields occurs, its expression is suppressed until after fission. This obviously contrasts with the sensory field arrangement in *Lumbriculus* (Fig. 2).

A second comparison can be made between the proximal influence of the ectopic head in *Lumbriculus* with those of grafted structures in other species. A body part or tissue, when grafted to an inappropriate site, is assigned a new positional context and could potentially influence neighboring tissue. Most research involving grafted body parts has been done on invertebrates such as hydra, snail, and annelids (Marcum et al., 1977; Bode & Bode, 1984; Vining & Drewes, 1985; Syed et al., 1992; Minobe et al., 1995). In general, the grafted tissues were found to survive and extend neurites and blood vessels into the host system, but the proximal effects of these grafts were indeterminate.

Methods described here provide a novel experimental paradigm which is different than that involved in the regenerative processes of either paratomy or grafting. The results provide insights into this worm's remarkable abilities of neurobehavioral plasticity, regenerative repair, and morphallatic reorganization of the central nervous system.

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Figure Legends

Fig. 1. Maps of touch-sensory fields for swimming and reversal in normal worms and group 1 worms with ablated ventral nerve cord. The brackets, labeled as “Reverse” and “Swimming,” indicate the sensory fields for body reversal and swimming in normal worms. Points labeled P1-P4 are designated points where touch stimuli were routinely delivered before and after ablation. The ablation site (arrow) was between P2 and P3 or well within the original sensory field for swimming. One day after ablation, touch at P1 and P2 evoked responses that were restricted to segments anterior to the ablation site, as indicated by Ra and Sa. Also at 2 d, touch at P3 and P4 evoked body reversal and swimming that were restricted to segments posterior to the ablation site, as indicated by Rp and Sp. Whole body reversal and swimming indicated with R and S were restored in some worms at 3 d and in all of group 1 worms by 8 d after ablation. The percentages of trials that elicited specific responses are shown at the four stimulus sites.

Fig. 2. Maps of MGF and LGF sensory fields of group 1 and group 2 worms before and after ablation. In normal worms (before ablation), the MGF sensory field is approximately 40% of the body length. The LGF sensory field is approximately 70% of the body length. There is a 5-10% overlap zone of MGF and LGF sensory fields. In group 1, at 8 d after ablation, both MGF and LGF sensory fields were configured as those normal worms. In group 2, by 8 d after ablation, there was emergence of an MGF sensory field that included the ectopic head, 1-2 segments anterior to the ectopic head, and 10-15 segments posterior to the ectopic head. In the remaining segments, sensory fields were similar to those of normal worms.

Fig. 3. Graph showing MGF and LGF conduction times across the ablation site at 0, 3, and 8 d after ablation in group 1 worms. The inter-electrode conduction distance was 6 mm, which included the entire ablation site. Each bar indicates the mean conduction time \pm SEM, n=14.

Fig. 4. Photomicrograph of ectopic head and other outgrowths from ablation site. A) A typical ectopic head. B) A large bulbous outgrowth with two finger-like projections. C) A rare case in which both head-like and tail-like outgrowths were formed. Scale = 1 mm.

Fig. 5. Maps of touch-sensory field for swimming and body reversal behavior in worms with an ectopic head. Note that at 3 and 8 d after ablation, responses to touch at P1-P4 were always partial body responses and never reverted back to the whole-body responses, as in group 1 worms. All abbreviations are the same as described in Fig. 1.

Fig. 6. Recordings of MGF and LGF spikes from two recording channels (Ch1 & Ch2) before and after ectopic head formation. A) In normal worms before surgery, touch at P1 evoked an all-or-none posteriorly conducted MGF spike detected sequentially at Ch 2 and Ch 1. Touch at P4 evoked an all-or-none anteriorly conducted LGF spike. B) At 3 d after ablation, touch at P1 evoked an MGF spike which conducted to Ch 2 but not through the ablation site. Touch at P4 evoked an LGF spike which was conducted to Ch 1 but not through the ablation site. C) At 8 d after ablation, touch at P1 evoked an MGF spike which was detected at both recording electrodes. Touch at P4 evoked an LGF spike also detected at both recording electrodes. However, there were noticeable delays in both LGF and MGF spikes when

compared with before ablation. Voltage scale = 100 μ V. Time scale = 1 ms.

Fig. 7. MGF and LGF spikes from two recording sites (Ch1 & Ch2) before and 8 d after ablation. A) Before ablation, touch at P4 evoked an anteriorly conducted LGF spike. Touch at P3 evoked a posteriorly conducted LGF spike. B) At 8 d after ablation, touch at P4 evoked anteriorly conducted LGF spike. However, touch at P3 evoked a pair of MGF spikes. The second MGF spike was followed by a large muscle potential which is indicative of MGF spiking activity (Drewes & Fournier, 1990). Voltage scale = 100 μ V. Time scale = 1 ms.

Fig. 8. MGF and LGF conduction times through the ablation site in group 2 worms at 0, 3, and 8 d after ablation. Spikes were not conducted across the ablation site at 3 d after ablation. Each bar indicates the mean conduction times \pm SEM, n=20.

Fig. 9. Frontal longitudinal sections of a worm with an ectopic head. A) This particular section shows a triangular confluence of the MGF (M) in anterior, posterior, and ectopic head segments. Note that the right half of the ventral nerve cord in the ectopic head curved to join the posterior portion of the main body, whereas the left half of the ventral nerve cord in the ectopic head curved to join the anterior portion of the main body. B) This particular section, from the same worm, shows the right LGF of the ectopic head (L) which curved to join the right LGF of the main body axis in the posterior portion of the worm. The left LGF of the main body was directly joined in segments anterior and posterior to the ablation site (not shown in this section). Scale = 50 μ m.

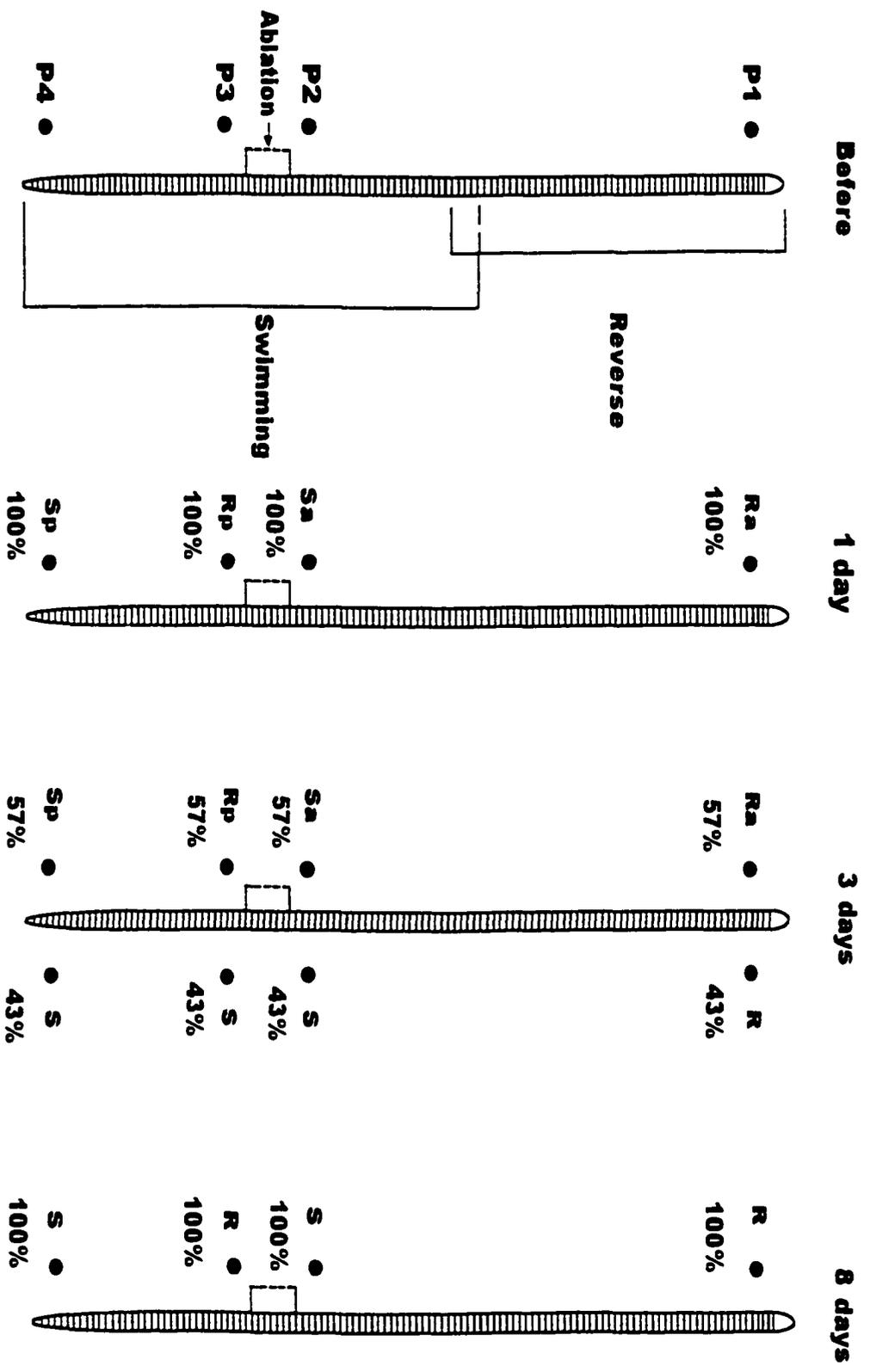


Figure 1.

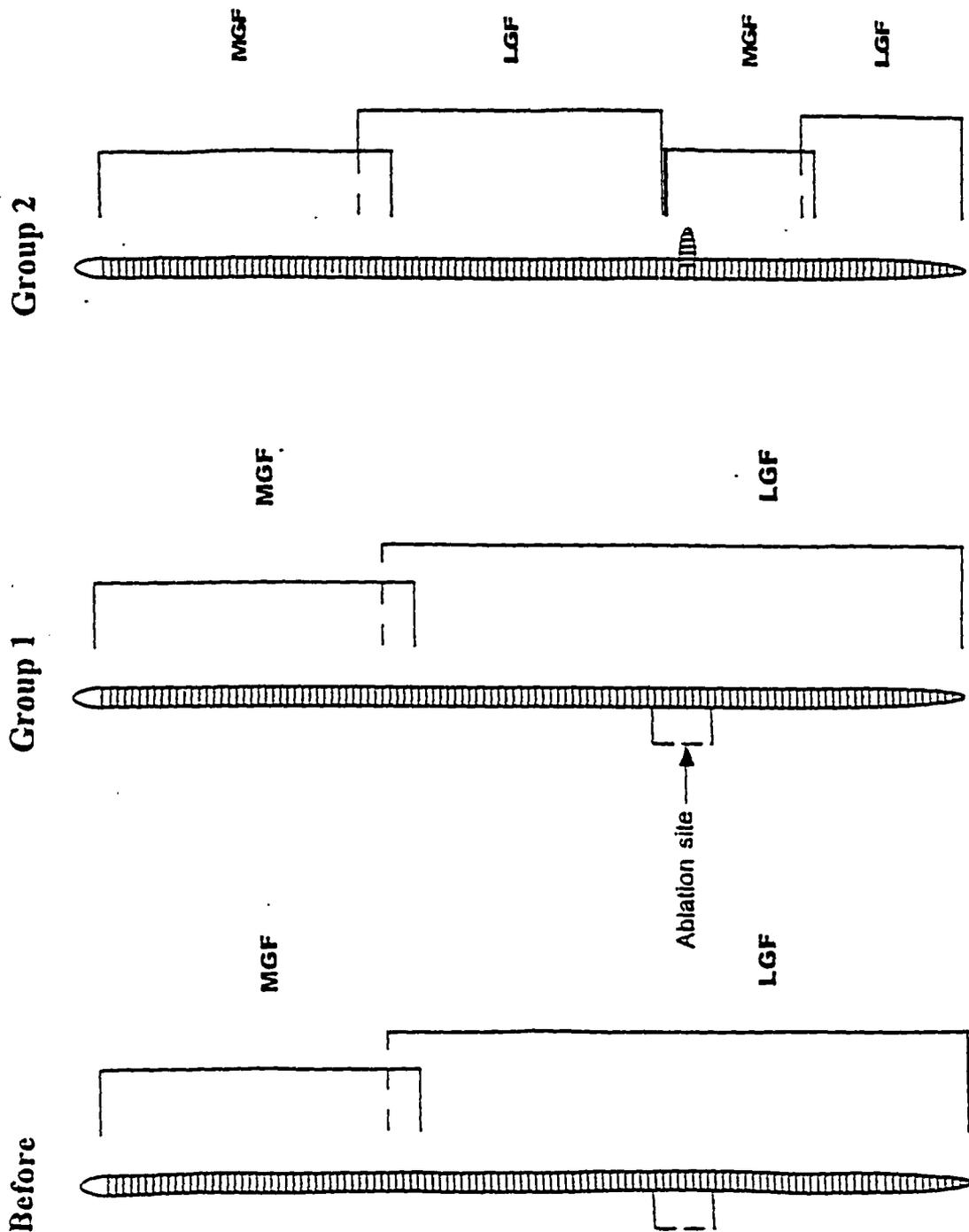


Figure 2.

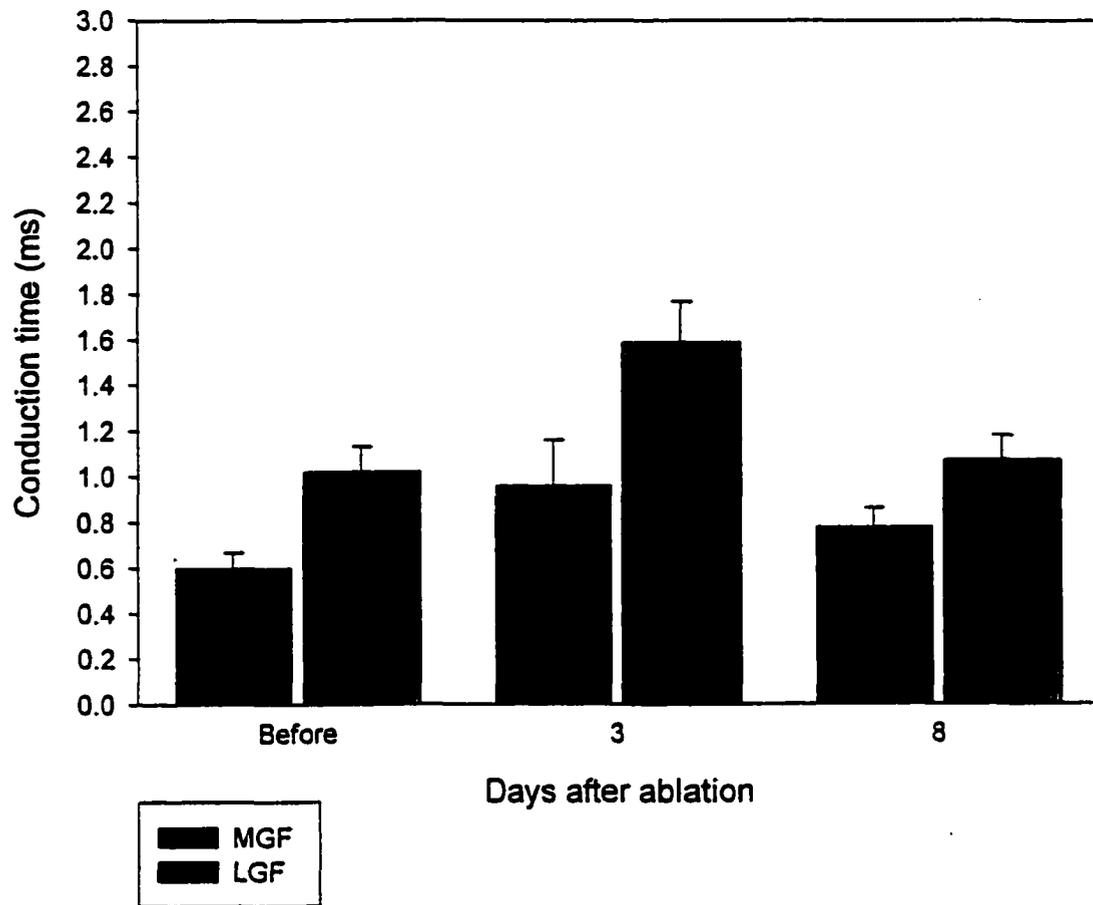


Figure 3.

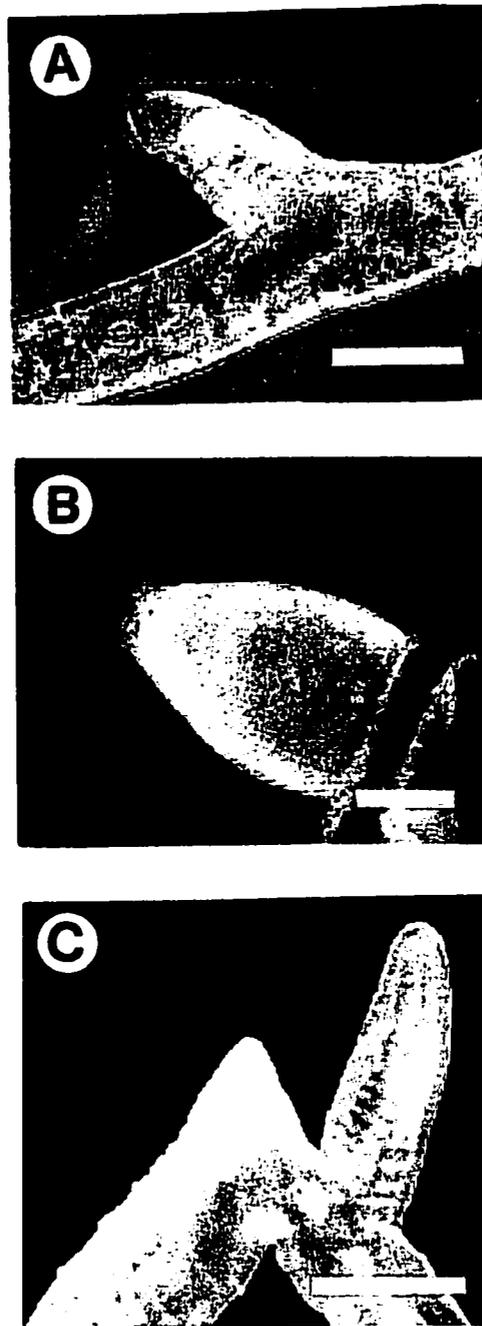


Figure 4.

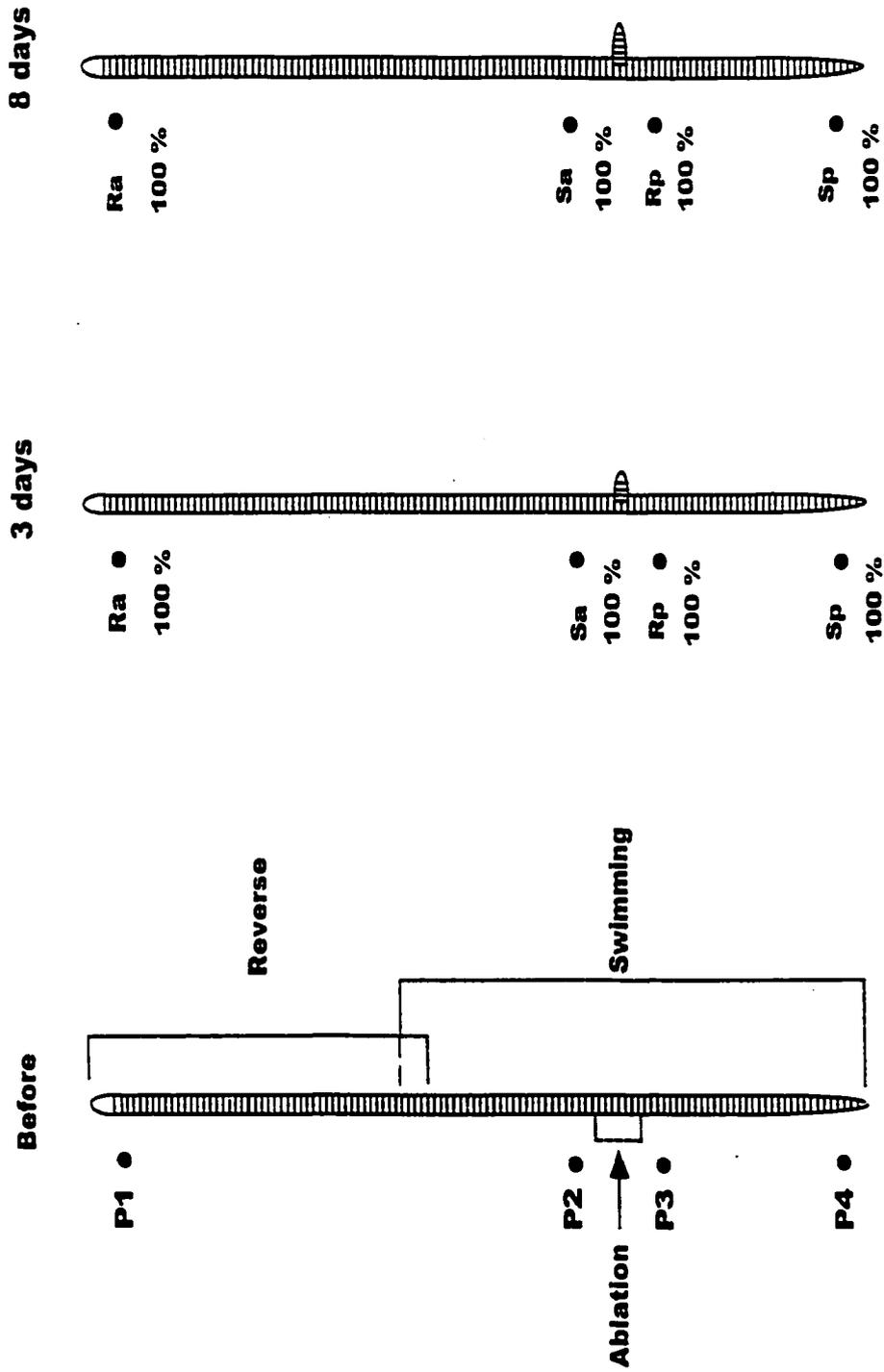


Figure 5.

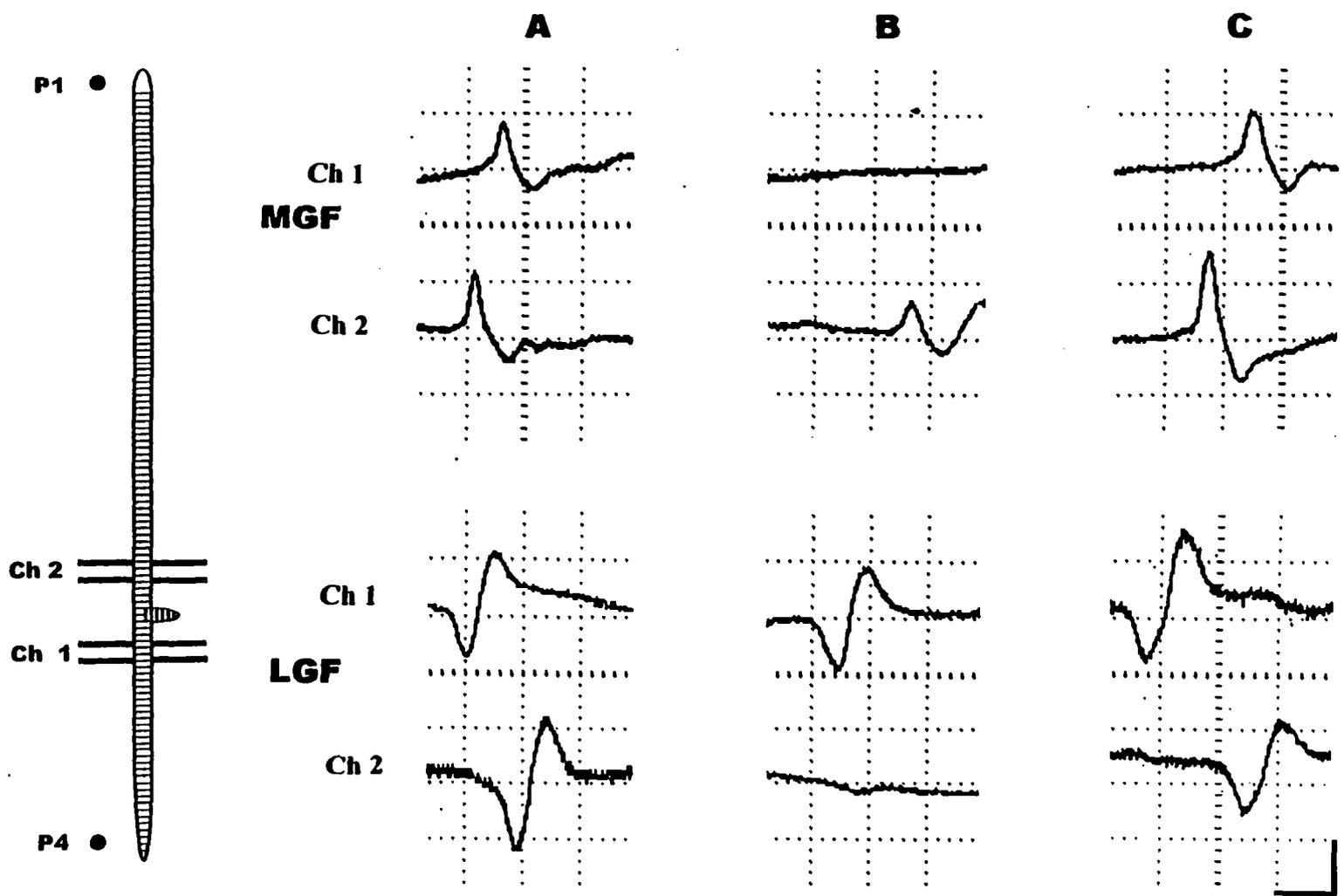


Figure 6.

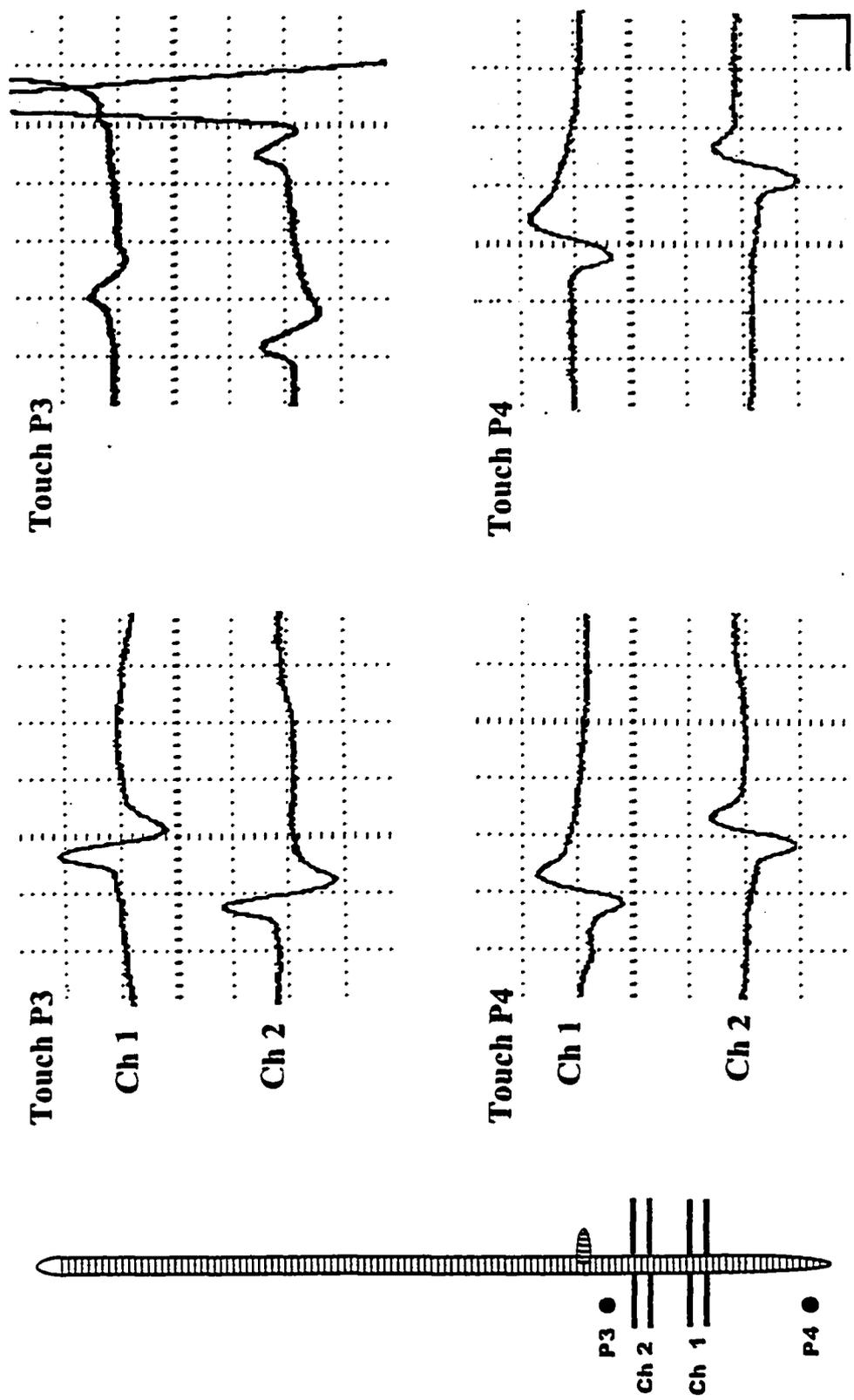


Figure 7.

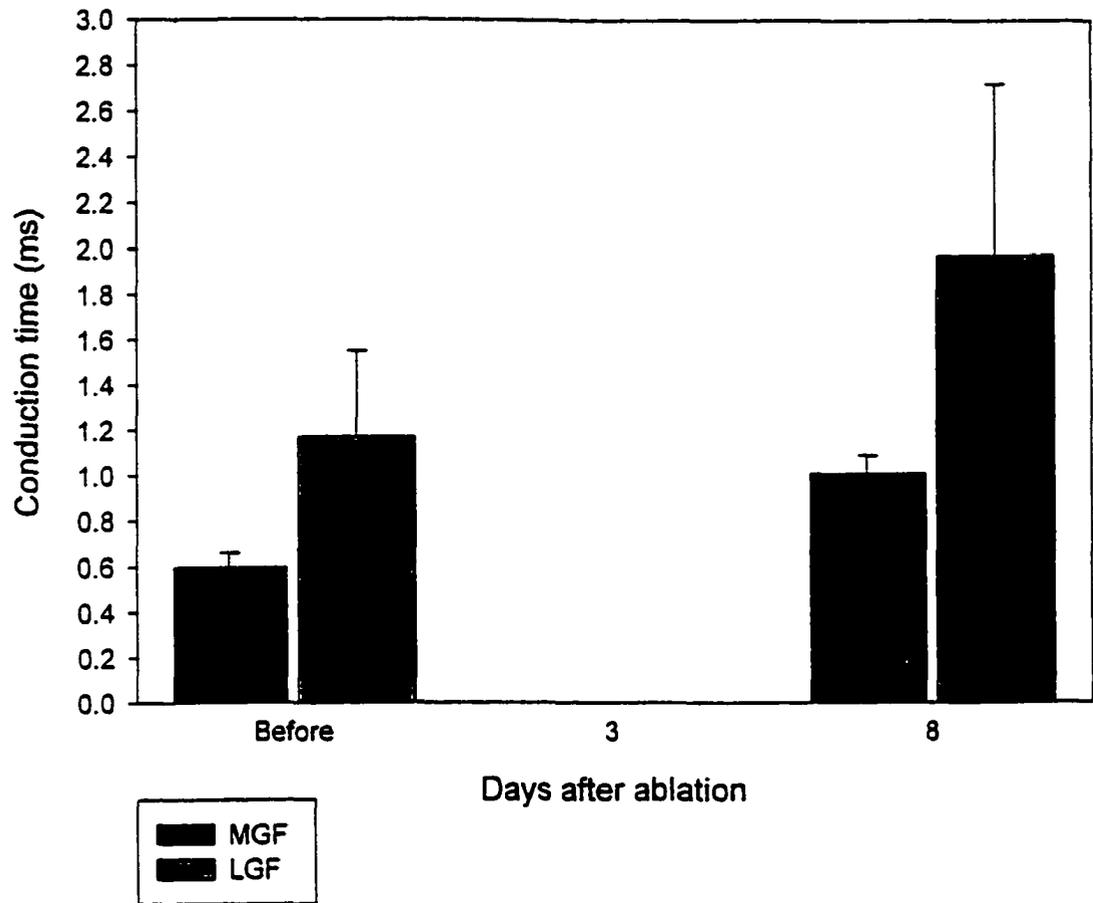


Figure 8.

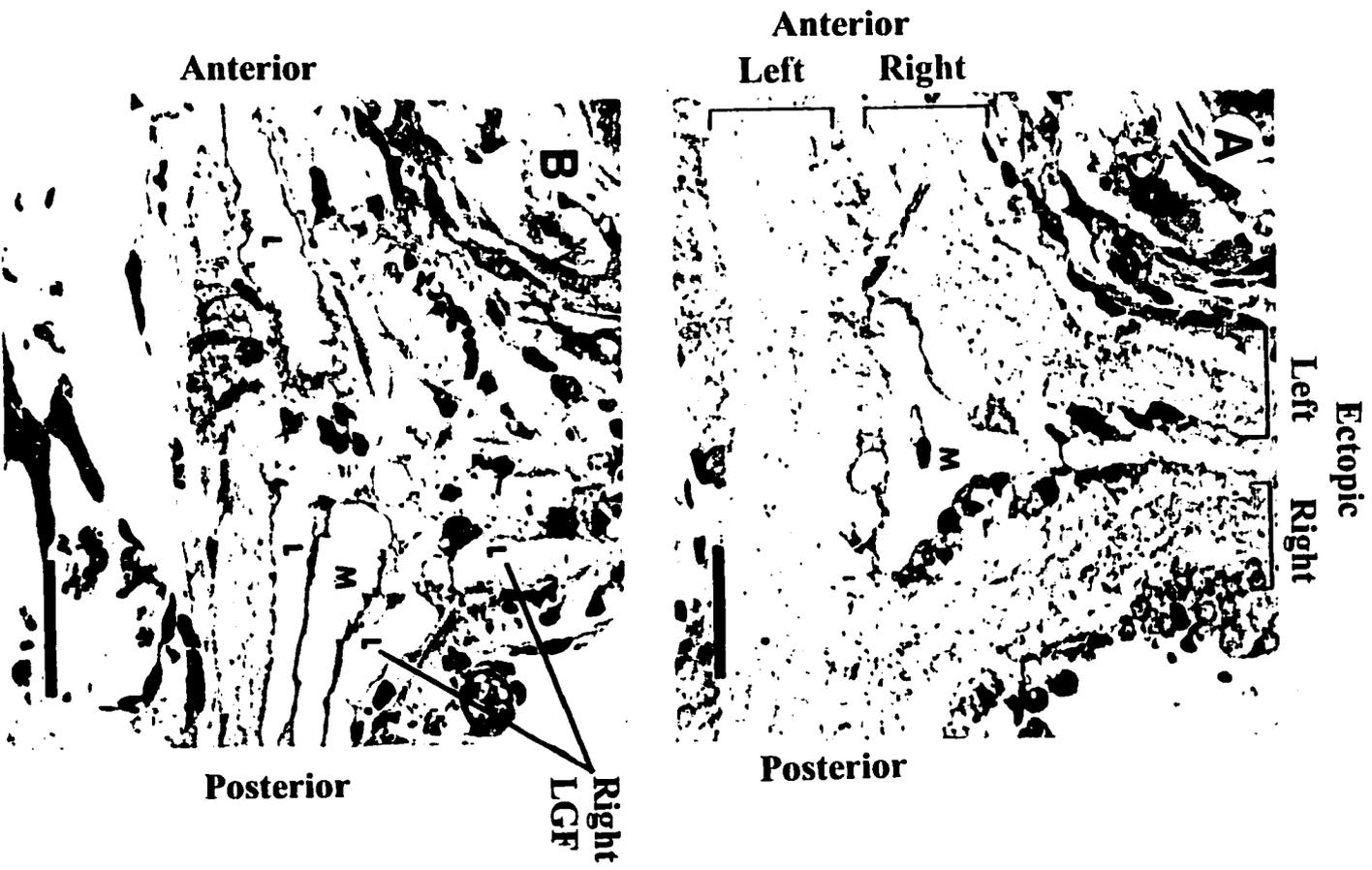


Figure 9.

CHAPTER 5. BLACKWORMS, BLOOD VESSEL PULSATIONS, AND DRUG EFFECTS

A paper published in *The American Biology Teacher*

Nalena M. Lesiuk & Charles D. Drewes

As biology teachers we want our students to understand and, if possible, witness first-hand the interactions and internal functions of organ systems in animals. As part of this experience, we also want them to appreciate the impact that biologically active substances, such as pharmacological agents (drugs) and toxins, have on these functions. By doing this, we hope they will gain insight and respect for the potent biological effects that such substances have in all animals, including humans.

Classroom activities involving circulatory functions are especially useful because student interest is readily captured by the rhythmic and vivid dynamics of blood pulsations in animals. Feasibility for such laboratory studies may be limited, however, by factors such as: (a) need to forcefully restrain and/or surgically invade animals in order to access the circulatory system, (b) time, special procedures, and/or apparatus required to measure pulsation rates under normal and treatment conditions, and (c) variability in results due to physiological stress related to handling and treatment of animals.

Our intent in this article is to introduce the freshwater oligochaete worm, *Lumbriculus variegatus* (common name: blackworms), as an organism that is well suited for classroom study of its closed circulatory system. We describe a set of simple, fast, non-invasive, and inexpensive methods for observing pulsations of the worm's dorsal blood vessel under baseline conditions and following treatment with two well-known pharmacological agents,

nicotine and caffeine. These methods should be widely adaptable for general biology and physiology courses at both high school and college levels.

Earlier versions of this exercise were field-tested during the summer of 1996 by high school biology teachers working at Iowa Lakeside Laboratory (West Lake Okoboji, IA) and at the Woodrow Wilson National Leadership Program for Teachers (Princeton, NJ). Much of the inspiration and initiative for this article arises from these teachers' success, enthusiasm, and support in working with this organism.

Background

Oligochaete worms have a closed circulatory system with bright red blood that resembles vertebrate blood. However, in these worms, the hemoglobin-like respiratory pigment, called erythrocrurin, is dissolved in plasma rather than in red blood cells (Jamieson, 1981). Two major blood vessels are present along the body of most oligochaetes. These are the dorsal and ventral blood vessels. In addition to these, there are lateral (commissural) vessels that connect the dorsal and ventral vessels in many segments.

In oligochaetes, blood is propelled forward by waves of muscle constriction that begin near the posterior end of the dorsal vessel. Usually, numerous waves are seen at different points of progress along the vessel. In the earthworm, *Lumbricus terrestris*, blood flow is augmented by pulsations in muscular lateral commissural vessels (so-called "hearts") found in segments 7-11. In the freshwater worm, *Lumbriculus variegatus*, there is one pair of commissural vessels in each anterior segment as far back as segment 18. All of these vessels are contractile and, along with the dorsal vessel, assist in pumping blood throughout the body (Stephenson, 1930). In addition, the dorsal blood vessel in most body segments

give rise to a pair of branched lateral vessels (Figure 1). These vessels are unusual in two aspects: they end blindly rather than connect with the ventral blood vessel and they are contractile. Contractions in these special vessels are synchronized with those of the dorsal blood vessel, presumably leading to more efficient pumping of blood throughout the body.

In earthworms, pulsation waves in the dorsal blood vessel pass the entire length of the worm but, occasionally, a wave fails to progress beyond the middle part of the body (Carlson, 1907). This means that pulsation rates may be somewhat higher in segments near the tail end than in segments near the head. The reported basal pulsation rate in the posterior segments of *Lumbricus terrestris* is 11 ± 2.1 SD beats per minute (Fourtner and Pax, 1971).

Regional differences in pulsation rates are more exaggerated in the blackworm, *Lumbriculus*. The basal rate toward the tail is much greater than in middle or head regions, indicating that many pulsation waves starting at the tail never succeed in traveling the whole length of the worm's dorsal vessel. Our data, from laboratory cultured worms, indicate that the basal pulse rate near the tail end ranges from 24-32 beats per minute while the basal rate in the middle of the worm ranges from 8-12 beats per minute (room temperature: 23° C).

As in many animals, pulsation rates in oligochaete worms are specifically affected by naturally occurring neurotransmitters (e.g., acetylcholine) and related pharmacological agents. In the earthworm, *Lumbricus terrestris*, acetylcholine significantly accelerates pulsation rate in the dorsal vessel. Nicotine, a drug that mimics acetylcholine in many situations, similarly accelerates pulsation rates of the dorsal vessel; at high concentrations, it may arrest pulsations altogether (Laverack, 1969). Such effects are striking and reliable but not readily observed without using restraint and surgical invasion. This makes it difficult to use earthworms as physiological models for student laboratories.

Given the actions of nicotine in earthworms, we asked whether similar effects may occur in smaller, freshwater species, such as blackworms, by merely immersing worms in nicotine solutions. Since the body wall is transparent, blood pulsations may be viewed directly and without surgical invasion, before and after short-term drug treatment.

In this report we introduce laboratory methods for rapid and reliable quantitation of basal pulsation rates in blackworms, *Lumbriculus variegatus*. We also describe simple treatment protocols for studying short-term effects of nicotine and caffeine on pulsation rates.

Materials Required

- Live *Lumbriculus variegatus* (common name: blackworm). Commercial sources include:
www.holidayjunction.com/aro/
www.novalek.com/korgdel.htm
www.carolina.com (Drewes, 1996).
- Dissecting microscope and light source (for use with paraffin block viewing chamber)
- Compound microscope (for use with Parafilm slide viewing chamber)
- Stopwatch timer (or a watch/clock with a second hand)
- Small glass or plastic containers for immersion of individual worms; about 20 ml volume per container; separate container for each worm (e.g., disposable plastic sauce cups from fast-food restaurants)
- Numerous 500 ml beakers for stock solutions and serial dilutions

- Disposable plastic pipette (or eyedroppers)
- Spring water (non-carbonated; most commercial brands will work)
- Nicotine (Sigma Chemical Co., St. Louis, MO)
- Caffeine (Sigma Chemical Co., St. Louis, MO or Carolina Biological, Burlington, NC)
- Parafilm
- Glass slides (25 mm x 75 mm x 1 mm)
- Hot plate (with low heat setting)
- Single-edge razor blade
- Jumbo paper clip (4.6 cm length; 1 mm diameter wire)
- Paraffin blocks (12.5 cm x 6.5 cm x 1.5 cm; five cakes per 16 oz box)
- Metal ruler
- Fine forceps
- Wooden applicator stick
- Straight human hair (2 cm long)
- Paper labeling tape
- A large finger bowl (for temporary storage of worms isolated from the colony)
- Cotton thread (preferably black)

Viewing Chambers

We designed two different, easy-to-make chambers for viewing dorsal blood vessel pulsations in *Lumbriculus*. Key features of chambers are: (1) a shallow and narrow trough

with dimensions slightly wider and longer than a single worm, so that the worm fits snugly into the chamber; and (2) a chamber with hydrophobic walls and hydrophilic floor.

The hydrophobic walls insure that the small volume of water, used to bathe the worm, coalesces within the trough as an elongate column bounded by its own surface tension. Thus, the worm tends to stay entrapped by the water surface tension. Worms also tend to stay in contact with the solid wall and floor surfaces of the chamber (thigmotaxis). This tends to minimize crawling activity and optimize viewing and counting of pulsations. We highly recommend taking time to make all viewing chambers prior to scheduled usage in class. The chambers can be rinsed and reused many times.

Parafilm Chamber

Cut off six rectangular pieces of Parafilm (6.5 cm x 1.5 cm) and remove the backing from each, making sure that the sheets are not wrinkled or stretched. Neatly stack the Parafilm sheets on top of one another and place this stack on a glass slide. Then, using a low-heat setting on the hot plate, heat the underside of the slide until the Parafilm just begins to soften and becomes slightly clear. [Caution: Do not overheat!]

Remove the slide from heat and quickly place a sheet of Parafilm backing paper on the Parafilm stack. Using your fingertips, apply gentle pressure to the backing and work any air bubbles that are under the Parafilm toward the edges of the slide. This step ensures bonding of the Parafilm sheets with each other and with the slide. If necessary, reheat briefly and repeat steps to ensure bonding.

Next, using a new, single-edge razor blade and a metal ruler, neatly trim excess Parafilm from around the edges of the slide. Remove trimmed edges with a fine forceps.

Then, using a sharp corner of the blade, cut a rectangular outline (2 mm x 50 mm) in the center of the Parafilm. Make sure that the razor blade slices through all six layers of Parafilm and that the blade is advanced a couple millimeters beyond each corner of the rectangle.

Then, use the forceps to remove the centerpiece of Parafilm, thus forming the trough.

A chamber with dimension shown in Figure 2 holds one worm and about 40 μ l of water. Trough size can vary slightly, but make sure it is not too wide or long. When worms have too much space to explore, the observer has less frequent opportunity for viewing and counting pulsations.

Paraffin Block Chamber

This is an easy-to-make and economical alternative to the Parafilm slide chamber. Each chamber is made from a block of household paraffin wax, normally used for canning or candle making. Open up a jumbo paper clip and position one end of it perpendicular to the paraffin block at the starting point shown in Figure 3. Then, press the tip of the wire gently into the wax and begin pulling it smoothly and repeatedly along the edge of a ruler for a length of 4 cm. The idea is to make a straight, shallow trough in the paraffin with final dimensions about 1 mm deep, 1 mm wide, and 4 cm long. Try to keep the depth of the trough uniform from one end to the other and carefully clean away excess wax scrapings.

Next, cut a length of cotton thread (preferably black) slightly shorter than the trough length. Moisten the thread with spring water and place it in the trough. Then, hold a glass slide perpendicular to the block and insert the short edge of the slide into the trough. Press the slide against the floor of the trough so that the thread is imbedded partially but not completely into the paraffin floor. The moist thread will create a hydrophilic surface for

water in the trough and a rough surface for worms to crawl upon. Each trough holds one worm and about 50 μ l of water. Two or three parallel troughs can be made in the same paraffin block, but troughs should be at least 1 cm apart.

Stock Solutions

1. Prepare a 10 mM stock solution of nicotine (mol wt: 162.2; density 1.0097) using 0.8 ml nicotine in 500 ml of spring water.
2. Use appropriate amounts of the 10 mM nicotine stock solution to make 500 ml quantities of each of the following dilutions: 1, 0.5, 0.25 and 0.01 mM. Label containers.
3. Prepare a 10 mM stock solution of caffeine (mol wt: 194.2) using 0.39 g caffeine in 200 ml of spring water.
4. Use appropriate amounts of the 10 mM caffeine stock solution to make the 500 ml quantities of each of following dilutions: 5, 3, 1, 0.1 and 0.05 mM. Label containers.
5. We recommend using solutions within 24 hours after preparation.

Experimental Design & Lab Groups

Divide the class into groups of three students. Designate one person as pulse observer, another as timer/calculator, and a third as data recorder. For groups of two students, one person is the observer and the other does both timing and data recording.

For each 45-60 minute block of class time, each group will have time to gather complete data sets (i.e., pulse rates before and after treatment) from about three worms. For

convenience, all three worms may be from the same treatment group. A recommended time-line for one group's activities over a 45 minute interval is shown in Figure 4.

With a class of 21-24 students, seven or eight groups may be formed, with each being assigned to one nicotine or caffeine concentration, or to zero concentration (control). Then, class data is pooled to study overall trends.

For nicotine, we suggest using at least these three concentrations: 0.05, 0.25 and 1.0 mM. Similarly, for caffeine, we suggest at least three concentrations: 1, 3, 10 mM. In addition, one or two student groups should be assigned as controls (zero drug concentration), in which all protocols are identical to the drug-treated worms, except that the "treatment solution" is pure spring water.

Procedures

Because *Lumbriculus* is a small, soft-bodied invertebrate with a relatively thin cuticle and epidermis, many pharmacological agents penetrate the skin and rapidly cause observable effects. To minimize variability and enhance clarity of results, we emphasize the following points related to chemical treatment and measurement of effects:

1. Selection and isolation: We prefer to use worms that are uniform with respect to overall body size and segmental dimensions. We avoid using worms with recently regenerated (immature and unpigmented) tail segments. We also recommend isolating worms overnight in a separate container (finger bowl) of clear spring water; this helps to clear the digestive tract.
2. Transfer and handling: Using a large-bore pipette, remove a worm from the finger bowl along with a little water. Deposit the worm in an observation chamber (either the

Parafilm slide or paraffin block chamber). Use a pipette to carefully remove excess water; the water level in the trough should be about even with the top surface of the Parafilm or paraffin block. Carefully remove any misplaced water with the corner of a paper towel or tissue. A cover slip is not needed or recommended. Let the worm crawl around for about 1-2 minute to adjust to confinement in the trough.

3. Viewing: Place the Parafilm slide chamber under lowest power of a light microscope. Observations should be possible with a minimum amount of transmitted light. For the paraffin block chamber, use the lowest power of a dissecting microscope.

4. Worm position: You may need to coax the worm to crawl into a position that allows easier viewing of the dorsal blood vessel. Crawling movements (forward or backward) are readily initiated by very lightly stroking the worm's tail or head with a hand-held flexible probe that will not damage worms. We highly recommend using the tip of a straight human hair to touch the worm and evoke crawling movements. The hair is attached by tape to an applicator stick, with about 1 cm of the hair extending beyond the tip of the stick.

5. Pulsation rates before treatment: Obtain a basal pulsation rate, from one specific body region, such as a mid-body location. Although you will see contractions of the dorsal vessel at multiple locations along the worm, concentrate your viewing on a single selected segment and count the number of pulsations that move through that particular segment over a 30 or 60 second interval. A stopwatch or watch with a second hand can be used for measuring the elapsed time. A count of 6 pulses in a 30 second time interval, for example, is a pulsation rate of 12 pulses per minute. Obtain an average pulse rate for each worm based on the mean of two to four separate rate counts.

6. **Transfer to treatment solution:** After obtaining a basal rate, use the pipette to add extra spring water to the trough, then remove the worm with the pipette and transfer it to a small container (20 ml) of treatment solution. The treatment time may be varied, but we recommend 15 minutes to observe full effects.

7. **Pulsation rates after treatment:** After treatment, rinse the worm briefly in spring water; this is important to prevent contamination of the viewing chamber. Place the worm back in the viewing chamber and measure pulsation rates as before. Make sure that measurements are made from the same body location used for pretreatment measures.

8. **Control groups:** Drug effects in treated worms must be compared to "effects" in a control group. Pulsation rates in the control group are determined initially (as in treatment groups) and then again after a 15 minute "treatment" during which worms are immersed in pure spring water.

9. **Comparisons:** Compute and compile mean pulsation rates, before and after treatment, for each worm, as shown in Table 1. Compute overall means, before and after treatment, for each group. Then, calculate the change in pulsation rate (rate after treatment minus rate before treatment) for each group. Graph these changes as a function of concentration to see general trends.

If desired, a variety of statistical applications may be applied to the pooled data; however, the "n" value (number of replicates) for each group should be at least five worms. Statistical significance between "before" and "after" values within a given group may be determined using a "paired difference" t-test.

Sample Results: Nicotine

Figure 5 shows typical changes in pulsation rate of the dorsal blood vessel after a 15 minute treatment with nicotine. All data was obtained from the middle part of the body. In the control group and in the lowest nicotine concentration (0.01 mM), we found no significant changes in the pulsation rate. Basal pulsation rates in these two groups were approximately 12 pulses per minute. However, at the next highest concentration, 0.05 mM, the pulsation rate increased sharply (about 70% increase above basal rates). This indicates that the "threshold concentration" was between 0.01 and 0.05 mM. Maximum effects may be expected at 0.25 mM, at which the rate increased to almost 150% above basal level. However, the pattern of nicotine effects changed dramatically at concentrations ≥ 0.5 mM. At this concentration, pulsations were completely blocked (rate = 0 pulse/min) in six of ten worms. In the other four worms, pulsation rates were very high. Thus, the net rate change for this group was near zero, but variability of effects was very large. Finally, at the highest concentration (1 mM), pulsations were absent in all 10 worms (rate = 0 pulse/min).

Besides effects on pulsation rates, other effects of nicotine occurred. At all concentrations above 0.05 mM, worms were immobilized and incapable of crawling in response to touch stimulation. Interestingly, all effects of nicotine are sublethal and reversible, provided that worms are allowed to recover in spring water after treatment.

In some biological systems (such as vertebrate skeletal muscle), nicotine is known to mimic the effect of the natural excitatory neurotransmitter, acetylcholine, by acting on acetylcholine receptors (nicotinic type). Studies in earthworm have shown that both acetylcholine and nicotine accelerate the pulsation rate of dorsal blood vessel and, at high concentration, completely arrest pulsations (Laverack, 1963). Similar nicotine effects are

shown here using the dorsal blood vessel in *Lumbriculus variegatus*. These results support the idea that there is a cholinergic excitatory control of the dorsal blood vessel pulsations in oligochaetes. The paralysis of worms that occurs during nicotine treatment is also expected, since excitatory synaptic transmission at the oligochaete neuromuscular junction is probably cholinergic (Jamieson, 1981).

Sample Results: Caffeine

Figure 6 shows typical changes in mid-body pulsation rates in worms treated for 15 minutes with various concentrations of caffeine. The threshold concentration is between 0.05 and 1.0 mM, while a 3 mM concentration causes a maximum increase in pulsation rate, about 70% greater than the basal rate. Unlike the effects of nicotine, there is no blocking of pulsations at higher caffeine concentrations. It is not clear whether the excitatory action of caffeine is the result of a direct or indirect effect.

Alternative Experiment

As an alternative to treatment with pure nicotine, water extracts from a tobacco product, such as cigarette or chewing tobacco, may be used. These extracts also have potent excitatory effects on pulsation rates.

As one possibility, we suggest placing the tobacco contents of one cigarette into 50 ml of warm spring water (about 60° C). Stir this continuously for 15 minutes. Then, filter the solution using standard filter paper and allow it to cool. Dilute this solution to 50%, giving a total of 100 ml of stock solution. The minimum amount of stock solution needed for

treatment of each worm is about 3 ml. Be sure that the treatment container is sufficiently small that worms are totally immersed in the solution.

Discussion

Few animals are suitable for studying effects of pharmacological agents on circulatory functions in a general biology laboratory. Typical problems one encounters are time limitations for data collection, need for special apparatus, and variability due to animal stress and trauma.

Among vertebrates, frogs and turtles are often used for studying pharmacological effects on cardiac function *in vivo*. Usually, this requires anesthesia (or pithing) and surgical invasion. However, many concerns exist regarding economic, ecological, and ethical aspects of using these animals and procedures in general biology laboratories.

Among invertebrates, *Daphnia* has been commonly used for observing heart beats *in vivo* and studying effects of temperature and drug exposure. However, *Daphnia* is not only difficult to immobilize, but the rapidity and variability in its heart rate make it difficult for students to obtain reliable and meaningful quantitative data. Further, *Daphnia* is not easily reared in the laboratory.

Blackworms, *Lumbriculus variegatus*, provide numerous advantages over *Daphnia*. First, they easily raised and handled in the laboratory. Second, it is easy to obtain pulse rate measures, both before and after drug exposure. Third, basal pulse rates are relatively slow, and exposure effects are robust and consistent from one worm to another.

However, as with any quantitative studies of biological rate functions, instructors will need to have a well planned strategy for students' data acquisition and analysis of results.

Decisions about group size and the total number of worms monitored by each group are important considerations. Also, trade-offs may be necessary regarding the number of compounds used, the number of concentrations per compound, and the number of worms in experimental and control groups.

Besides convenience in studying circulatory physiology, blackworms offer unique possibilities for investigating other areas of organismal biology including regeneration of amputated head and tail segments, biomechanics and behavior of crawling and swimming, and neurophysiological recording from giant nerve fibers. We are currently developing and documenting each of these as routine exercises for general biology laboratories. The authors would be pleased to share ideas, information and experience regarding any of these.

Acknowledgment

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Figure legends

Figure 1. Side view of several body segments showing major blood vessels and internal

Anatomy of *Lumbriculus*. A pulsation wave (PW) of the dorsal blood vessel (DBV) is shown. Note that contraction of the dorsal blood vessel is synchronized with contraction of the blind lateral vessels (LV). VNC, ventral nerve cord; VBV, ventral blood vessel; I, intestine.

Figure 2. Parafilm slide viewing chamber. Six layers of Parafilm are bonded by heat to a glass slide and a trough is made by cutting the Parafilm with a razor blade. Trough dimensions are: 1 mm width, 1 mm depth, and 4 cm length.

Figure 3. Paraffin block viewing chamber. A trough is made using the end of a paper clip to chisel a groove 1 mm wide, 1 mm deep, and 4 cm long in the wax.

Figure 4. Suggested time-line of activities for one student group over a 45 minute interval.

Figure 5. Dose-response graph showing effects of different nicotine concentrations on pulse rates in *Lumbriculus*. Each point represents the mean change in rate for 10 worms (four measures of rate per worm). Vertical bars indicate ± 1 S.E.M. The horizontal line at 0 indicates the average basal pulsation rate (≈ 12 pulsation/min). The maximal increases occurred at 0.25 mM nicotine. At 1.0 mM, pulsations were completely abolished (i.e., pulsation rate decreased from 12 to 0 pulse/min).

Figure 6. Dose-response graph showing effects of different caffeine concentrations on pulse rate in *Lumbriculus*. Each point represents the mean change in rate for 10 worms (four measures of rate per worm). Vertical bars indicate ± 1 S.E.M. The horizontal line at 0 indicates the average basal pulsation rate (12 pulsation/min).

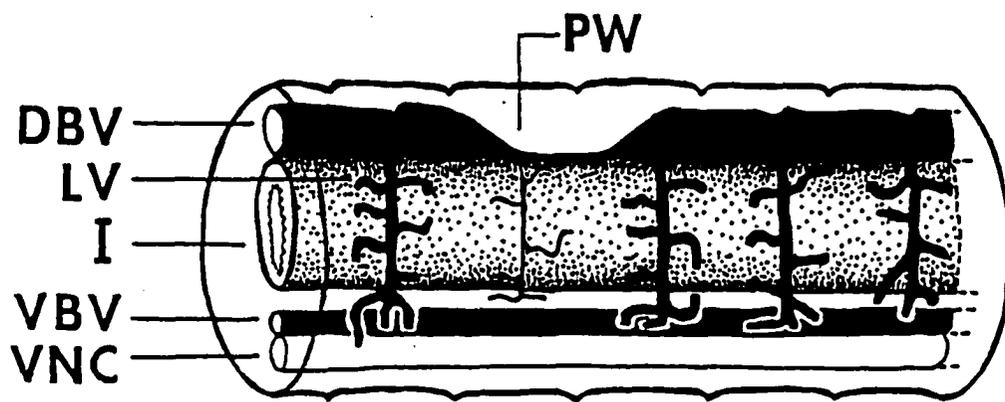


Figure 1.

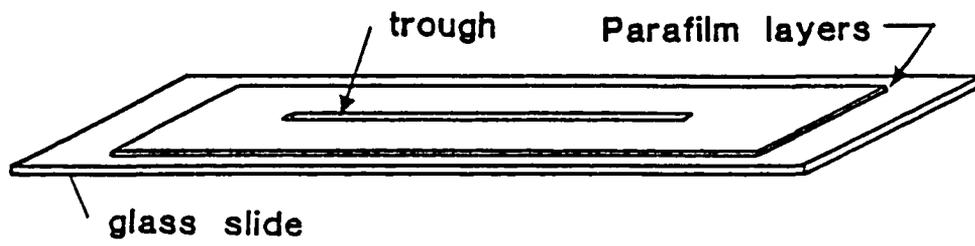


Figure 2.

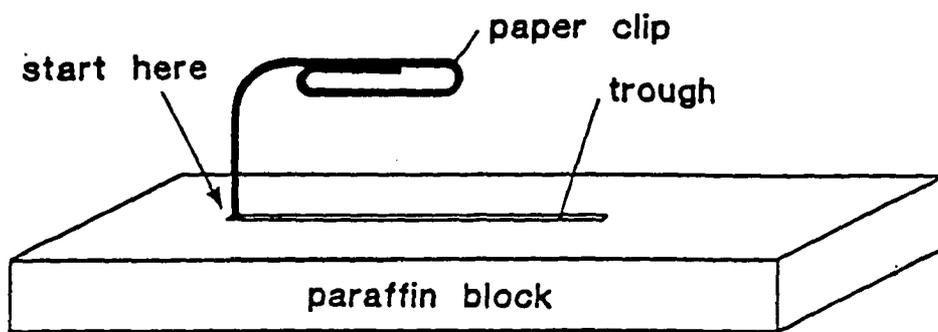


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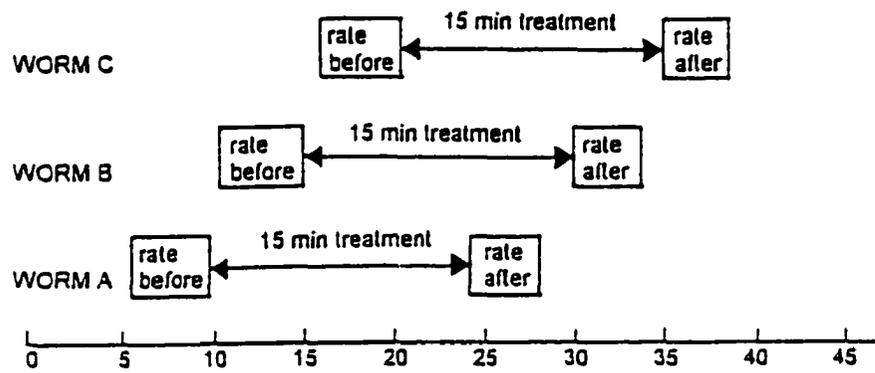


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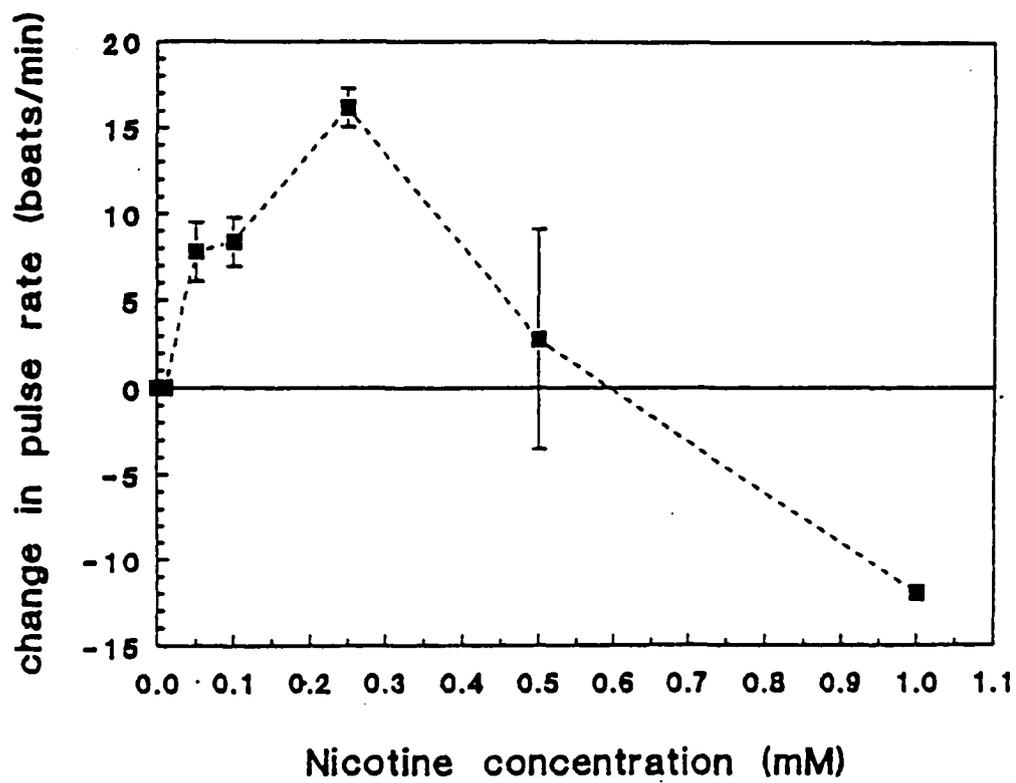


Figure 5.

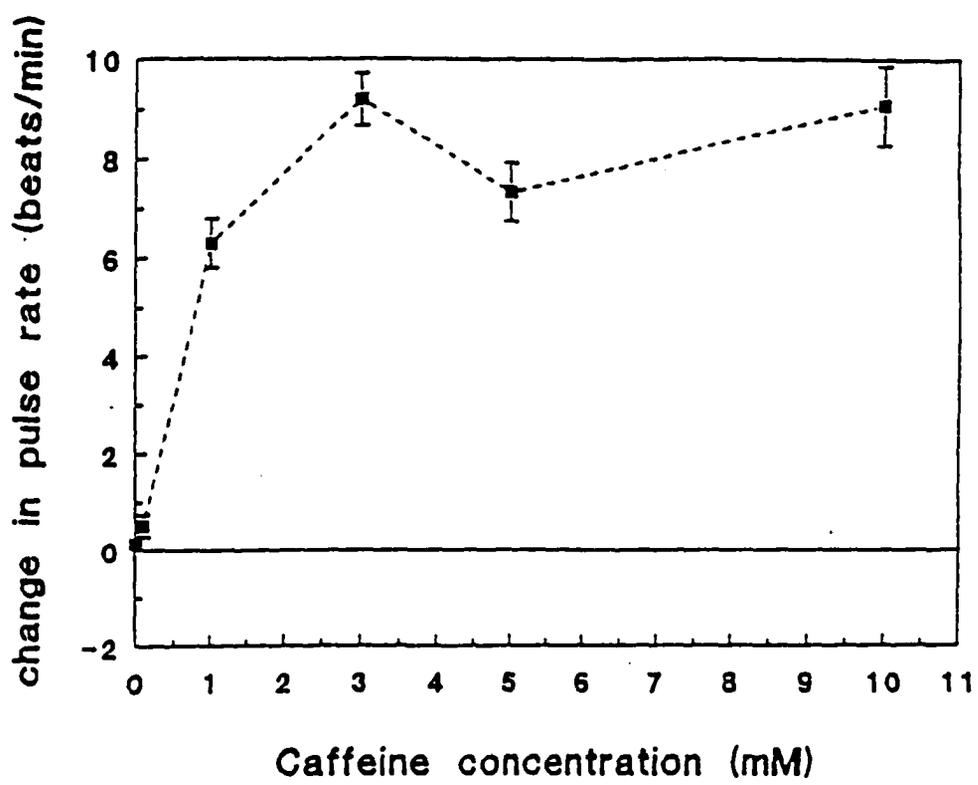


Figure 6.

Table 1. Format of data sheet and sample data for one worm.

Student's name: _____ Date: _____
 Compound used: _____ Concentration: _____ mM
 Data sheet

	Rate Before Treatment (beats/min)	Rate After Treatment (beats/min)	Change in pulse rate (beats/min)
Worm A rate 1 rate 2 rate 3			
mean A			
Worm B rate 1 rate 2 rate 3			
mean B			
Worm C rate 1 rate 2 rate 3			
mean C			
MEAN			

Sample Data for nicotine treatment (0.05 mM)

	Rate Before Treatment (beats/min)	Rate After Treatment (beats/min)	Change in pulse rate (beats/min)
Worm A rate 1 rate 2 rate 3	12 10 12	19 22 20	
mean A	11.3	20.3	9.0

CHAPTER 6. GENERAL CONCLUSION

Lumbriculus variegatus is known to have external morphological, microanatomical, physiological, and behavioral gradients along its body axis. The advantages of using this worm in studying these gradients include the possibility of performing behavioral assessment of unique responses, swimming and body reversal, and non-invasive electrophysiological recordings of the giant nerve fiber impulse conduction. Because all these methods are non-invasive, the changes in the same worm can be studied repeatedly both before and after experimental manipulations.

Although these four papers in this thesis focus on different areas, there are two commonalities that relate them. The first common theme relates to segmental gradients in physiology and behavior. Here are some conclusions from these papers that relate to segmental gradients.

The autotomy reflex in *Lumbriculus* is a stereotyped, all-or-none rapid response in which the body is clearly and quickly separated into two portions. The direct relationship between the amount of compression and the probability of autotomy was evident. In addition, when we used the same amount of compression, we found that the probability of autotomy in the tail was higher than in the middle region of the worms. This clearly shows that there is segmental gradient with respect to the ability to autotomize.

The segmental gradients were also evident in both the transection and ablation experiments. A characteristic behavioral response (i.e., body reversal) that is normally evoked only by stimulation of anterior segments can be transiently acquired by posterior segments immediately after transection and ablation. Furthermore, the presence of an ectopic

head can exert permanent influences on sensory field and behavioral response patterns along the longitudinal body axis.

Our results showed that there is a segmental gradient with respect to the blood pulsation rates in the middle and tail regions of the worm. The baseline pulsation rate increases from anterior to posterior regions with basal rate in the midbody of 8-12 beats/min and tail region of 24-32 beats/min.

The second theme of this thesis related to effects of nicotine on the worm. Nicotine, a cholinergic agonist, was found to be a dependable and powerful immobilizing agent that also blocked autotomy responses, thus enabling us to perform surgical manipulations of the ventral nerve cord in *Lumbriculus*. This nicotine effect is temporary and it is completely reversible within 3 h of recovery.

Nicotine has a profound effect on blood vessel pulsations of *Lumbriculus*. Low concentration of nicotine accelerates the pulsation rate and at high concentration blocks pulsations completely. Such effects of nicotine were completely reversible. The effects of nicotine and its use as an immobilizing agent in *Lumbriculus* enabled us to perform invasive surgical procedures that had never been done before. The results yielded experimental outcomes that provide new insights about neural plasticity and reconnection in this organism.

Finally, a new educational model for studying blood pulsations within a closed circulatory system is introduced. This model has already been incorporated into biology classes in both high school and college levels in the United States.

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