1984

Development and regeneration of the escape reflex in the earthworm, *Eisenia foetida*

Elizabeth Platt Vining

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DEVELOPMENT AND REGENERATION OF THE ESCAPE REFLEX IN THE EARTHWORM, EISENIA FOETIDA

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Development and regeneration of the escape reflex
in the earthworm, *Eisenia foetida*
by
Elizabeth Platt Vining

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
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## INTRODUCTION

- Pretesting Procedure
- Transplantation
- Electrophysiological Procedures
  - Non-invasive Recordings
  - Intracellular Recordings

## RESULTS

- Recovery of Giant Fiber Through-conduction
- Recovery of Sensory Function
- Recovery of MGF-mediated Motor Responses
- Behavior
DEDICATION

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ABSTRACTS (*Name of presenter)


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GENERAL INTRODUCTION

Nervous system development involves nerve cell proliferation, migration, establishment of appropriate functional connections with target cells, and other maturational changes. Traditionally, two approaches have been used to investigate neuronal development. The first involves the study and manipulation of neurons during their normal development. The second involves interruption of established neuronal connections and subsequent study of regeneration or other compensatory responses. Both approaches have been used in this dissertation to investigate various aspects of nervous system development and regeneration in the earthworm, *Eisenia fetida*.

Some advantages of using the earthworm nervous system include the existence of identifiable neurons and the capability for non-invasively recording spike currents from these neurons (Drewes and McFall, 1978; O'Gara et al., 1982). Advantages of using this particular species include rapid growth and regeneration.

Despite numerous advantages, there have been relatively few studies of nervous system development in earthworms and these were based solely on morphological or behavioral observations (Kleinenberg, 1879; Prosser, 1933; Ogawa, 1939). Recently electrophysiological data obtained from the developing nervous system in earthworms (O'Gara et al., 1982). In this study, the authors recorded giant fiber electrophysiological activity in embryonic and postembryonic worms and described
time-dependent increases in giant fiber diameter and conduction velocity.

There have been several studies that demonstrate the regenerative capacity of the earthworm nervous system, the earliest being those by Bovard (1918) and Yolton (1923). They noted recovery of rapid escape responses approximately one week after ventral nerve cord (VNC) transections. More recently, electrophysiological properties have been described for regenerating giant fibers after VNC transection in Lumbricus terrestris (Birse and Bittner, 1976; Balter et al., 1980; Birse and Bittner, 1981). In these investigations, giant fiber regeneration was specific and conduction time of the giant fiber spikes was markedly reduced in the lesioned area.

Part I of this dissertation is an investigation of regenerating giant fibers in the earthworm, Eisenia foetida, following VNC transection and grafting of body segments. Parts II and III are investigations of the morphology and electrophysiology of central and peripheral connections between transplanted VNC and recipient target tissues. Part IV is an investigation of food deprivation effects on postembryonic development of giant nerve fibers.

Explanation of Dissertation Format

This dissertation is written in the alternate format. Each major division (Parts I-IV) is a complete manuscript modified to conform with the specifications of the Iowa State University Thesis Office. Each part has its own introduction, materials and methods, results,
discussion, and references. A general summary of the entire dissertation follows these four parts.
PART I. REGENERATION OF GIANT FIBERS FOLLOWING VENTRAL NERVE CORD TRANSECTION AND GRAFTING IN THE EARTHWORM, EISENIA FOLETIDA
INTRODUCTION

The giant fibers of annelids are particularly advantageous for studies of nerve regeneration because they contain a relatively small number of identifiable neurons and have a high capacity for regeneration. In the case of earthworms, early investigators (Bovard, 1918; Hall, 1921; Yolton, 1923) noted behavioral and morphological signs of prompt regeneration of giant fibers following transection of the ventral nerve cord. More recently, electrophysiological properties of regenerating medial (MGF) and lateral (LGF) giant fibers in the earthworm, *Lumbricus terrestris* have been described (Birse and Bittner, 1976; Balter et al., 1980; Birse and Bittner, 1981). These investigators concluded that giant fiber regeneration involved mechanisms of either fusion or formation of an electrical synapse between proximal and distal stumps. In fact, these appear to be the two main mechanisms of regeneration when both proximal and distal stumps survive (Hoy et al., 1967; Bittner and Johnson, 1974; Kennedy and Bittner, 1974; Carbonetto and Muller, 1977).

In this study, the electrophysiological and morphological properties of regenerating giant fibers were examined following ventral nerve cord transection in the earthworm, *Eisenia fetida*. This species is easily reared and capable of rapid growth and development (Kaplan et al., 1980; Neuhauser et al., 1980). In addition, properties of regeneration were compared for transections at anterior versus posterior segmental levels. Segmental variations in these properties might be
expected in view of electrophysiological and morphological data suggesting longitudinal gradients in synaptic inputs and outputs of the giant fibers (Gunther and Walther, 1971; Gunther, 1973; Gunther and Schurmann, 1973; Drewes and McFall, 1980; Smith and Mittenthal, 1980).

Successful connection of transected giant fibers within a given animal raises the possibility that comparable connections may occur in apposed nerve cords from different animals. Numerous classical studies have demonstrated that portions of two different worms can be successfully joined by the end-to-end grafting of whole body segments (Joest, 1897; Morgan, 1897; Hazen, 1899, Morgan, 1901; Korschelt, 1931). However, it has not been determined if the nervous systems of such grafted preparations form functional connections with one another and to what degree these connections are similar to those of transected giant fibers.
MATERIALS AND METHODS

Nerve Cord Transection

Mature earthworms (*Eisenia foetida*) were obtained from laboratory cultures (O'Gara et al., 1982), rinsed in distilled water, anesthetized on ice for approximately one minute, and restrained ventral side up with foam rubber pads. In one experimental group (n = 20), a 1.5 mm longitudinal incision was made just through the body wall at segments 25-26 (anterior transection). In a second group (n = 20), a similar cut was made at segments 75-76 (posterior transection). The exposed ventral nerve cord (VNC) was then completely severed with a fine scissors between segmental nerve I and II-III, care being taken to avoid puncturing the gut or ventral blood vessel. The cut ends of the VNC separated by approximately 0.5 mm when severed. Each animal was then placed in a glass petri dish filled with moist dirt and manure (one animal per dish) and allowed to recover at 27°C. Recovery of giant fiber spike through-conduction was tested *in vivo* using a printed circuit board grid for obtaining non-invasive electrophysiological recordings at multiple sites (O'Gara et al., 1982). Animals were tested 16 h after transection and at four hour intervals thereafter. All testing was done at room temperature (22°C). Each testing period lasted between one and two hours. Between tests animals were returned to their individual petri dishes at 27°C.

The lability of MGF spike conduction across the transected VNC was tested in preparations consisting of the last 40 segments of the worm,
rather than whole worms. These preparations proved especially useful because they 1) could be handled without inadvertently evoking MGF spikes, 2) exhibited little crawling activity, and 3) can survive for months without significant regeneration of body segments and without impairment of giant fiber conduction properties. After amputation from adult worms, the posterior preparations were placed in moist soil at 27°C for two days, allowing the cut ends to heal. Then, the VNC was transected at approximately segment 85 and the preparation was returned to its dish at 27°C.

Spike conduction across the transection was tested by placing each preparation ventral side down on a recording grid. A Sylgard block with a shallow groove was placed over the worm to restrict body movements. Electrical stimuli via the grid were used for direct stimulation of the MGF. The MGF spikes were readily distinguished from LGF spikes on the basis of spike waveform and conduction velocity (O'Gara et al., 1982). Threshold intensity for MGF was typically 5-7 V (0.025 ms duration), which was approximately 0.5 V less than LGF threshold.

The MGF responses to electrical stimulation at 20 Hz were recorded simultaneously from sites anterior and posterior to the lesion. The time from the beginning of stimulation, when MGF spikes were reliably conducted across the transection, to the first failure of spike propagation was determined with a stopwatch. The time required for subsequent recovery of conduction across the transection was monitored by testing with single pulse stimulation every five minutes.
Grafting

A 35 segment portion was removed from each end of a worm, the remaining middle portion being discarded. Two anterior or two posterior portions, from different worms, were then placed on a cold block of dental wax and positioned so that the two cut faces were apposed and the ventral nerve cords (VNC) aligned. Each portion was secured ventral side up with minuten pins inserted through the lateral edge of the body wall. The ends were then sewn together with 6-0 silk glued to a bent minuten pin. The first two stitches were made through the body wall on each side of the VNC. Each stitch included at least two segments of body wall from each end because the ends tended to pull apart if shorter stitches were used. The preparation was then unpinned, turned dorsal side up, secured again, and three or four additional stitches were made. After grafting, the estimated distance between the severed ends of the VNC was approximately 1.0 mm. Grafted preparations were then placed into individual petri dishes and covered with moist dirt.

Successful grafts, in which there was physiological evidence for giant fiber through-conduction, were obtained in 13 preparations, all of these involving posterior-posterior grafts. The 19 remaining posterior-posterior grafted preparations either pulled apart with each piece surviving (n = 6) or the entire preparation died (n = 13). No successful grafts were obtained in 24 preparations involving grafts of the anterior ends. In seven of these anterior-anterior grafts, the animals pulled apart as a result of vigorous crawling. The remaining 17
anterior-anterior preparations were connected but both ends were necrotic.

In successfully grafted preparations, non-invasive testing of giant fiber conduction was done at 24 h after grafting and every 12-24 h thereafter. Grafted preparations were maintained at 27°C and tested at room temperature (22°C).

Intracellular Recordings from Giant Fibers

Intracellular recordings of giant fiber responses to touch were obtained from normal animals in which 30 segments of VNC were exposed (Fig. 1) and bathed in worm saline (Drewes and Pax, 1974). Hook electrodes were placed under the remaining intact segments to simultaneously obtain surface recordings of giant fiber activity. Aluminum-silicate glass microelectrodes were filled with 3 M KCl and had resistances ranging from 20-60 Mohms.

Histological Preparation

Prior to fixation animals were anesthetized on ice, pinned ventral side down on cold dental wax, and dissected under saline. The VNC was exposed for approximately two segments on both sides of the transected or the grafted region. Four segments of VNC along with attached body wall were removed, pinned to a thin slab of Sylgard, and immersed overnight in 2.5% glutaraldehyde in 0.1 M cacodylate buffer at 5°C. Following fixation, the lateral edges of body wall were trimmed away leaving a strip of body wall approximately 5 mm along with the VNC.
Then, the strip was pinned to a Sylgard slab, dehydrated in a graded series of ethanol, cleared in two changes of xylene, and embedded in paraffin. Serial cross sections (5 μm thick) were stained with 0.5% toluidine blue in 0.5% borax.
RESULTS

Identification of Giant Fiber Spikes

Figure 1 shows correlated intracellular and non-invasive recordings of MGF and LGF spiking in response to touch. The amplitude of intracellularly recorded MGF spikes ranged from 70-80 mV (mean $E_{rest} = -67$ mV, range: -60 to -78 mV, $n = 26$) and LGF spikes from 60-75 mV (mean $E_{rest} = -62$ mV, range: -60 to -72 mV, $n = 8$). Giant fiber spike duration was approximately 2-3 ms. Each MGF spike was correlated with a stereotyped three-component waveform in the extracellular non-invasive recordings. The three components were essentially identical to those described by O'Gara et al. (1982) and consisted of a monophasic MGF spike, followed after 1.2 ms by a presumed motor neuron spike and muscle potential. Each LGF spike was correlated with a single biphasic spike in non-invasive records.

Initial Recovery of Giant Fiber Through-conduction

As expected, giant fiber spikes initially failed to conduct across the VNC transection (Figs. 2B and 3B). In whole worm studies, recovery of MGF through-conduction generally preceded recovery in the LGF. With anterior transections (segment 25), recovery of MGF conduction across the transection occurred 38 h ± 10 SD after transection. By comparison, recovery of LGF conduction occurred 48 h ± 16 SD after transection. The earliest recovery time for MGF or LGF was 20 h ($n = 5$). Although MGF and LGF recovery times were significantly different in anterior transections ($p < 0.05$), there were no such differences in posterior
Figure 1. Simultaneous recordings of intracellular and surface electrical activity from the giant fibers. A) Two monophasic MGF spikes (dots), evoked by light tactile stimulation (ST\textsubscript{ant}) of the head, were detected by hook electrodes (REC\textsubscript{ex}) in contact with the ventral surface. Each MGF spike was followed after 1.2 ms by a presumed motor neuron spike and muscle potential (O'Gara et al., 1982). The MGF spikes were detected more posteriorly by intracellular recordings (REC\textsubscript{in}) from exposed VNC. B) An all-or-none biphasic LGF spike, evoked by light tactile stimulation (ST\textsubscript{post}) of the tail, was recorded by hook electrodes (REC\textsubscript{ex}) on the ventral surface and more anteriorly by an intracellular electrode (REC\textsubscript{in}).
Figure 2. Simultaneous recordings of MGF spikes from six sites before and after severing the VNC at segment 75.  

A) Before severing the VNC the MGF spike, evoked by light tactile stimulation to the head, was conducted posteriorly at 7.7 m/s. The spike was followed by a motor neuron spike and muscle potential. 

B) By 0.5 h, after severing the VNC, the MGF spike was detected at three sites anterior to the transection but not posterior to the transection.  

C) By 20 h, in the same animal, the time across the transection was approximately 2.4 ms. The vertical bar in (B) and (C) indicates the location of the transection relative to the recording sites. Abbreviations: A—anterior, P—posterior.
Figure 3. Simultaneous recordings of LGF spikes from six sites before and after severing the VNC at segment 75. A) Before severing the VNC two LGF spikes, evoked by tactile stimulation to the tail, were conducted anteriorly at 3.9 m/s. B) By 0.5 h after transection, the two LGF spikes were detected at three sites posterior but not anterior to the transection. C) By 42 h, in the same animal, LGF spikes were conducted successfully across the transection. The conduction time of the spike across the transection was approximately 1.9 ms. The vertical bar in (B) and (C) indicates the location of the transection relative to the recording sites. Abbreviations: A— anterior, P— posterior.
transections (MGF mean = 40 h ± 11 SD, LGF mean = 46 h ± 14 SD). All these recovery times were much faster than those of transected giant fibers in Lumbricus terrestris (5-10 days for MGF; 12-18 days for LGF; Balter et al., 1980).

Through-conduction of LGF spikes across the graft (Fig. 4A) occurred in 13 preparations after a mean time of 65 h ± 26 SD. Through-conduction of MGF spikes across the graft (Fig. 4B) occurred in only six of 13 preparations but not until 7-14 days after grafting. In the remaining seven preparations, MGF through-conduction did not occur over three weeks of testing.

Initially, spike conduction across transected as well as grafted VNC was occasionally intermittent in one or both directions. Such intermittent conduction involved occasional failures of single spikes to conduct across the transected or grafted region. Also, during spike trains the first one or two spikes occasionally failed, but all subsequent spikes in the train were reliably conducted across the site. Similar phenomena involving intermittent conduction were observed by Balter et al. (1980) in regenerating giant fibers in Lumbricus terrestris.

Directionality of Conduction

In 31 of 40 animals with transected VNC, through-conduction of MGF and LGF spikes was initially reliable in either direction. In the other nine worms, however, through-conduction was initially unidirectional. For example, in anterior transections initial MGF conduction occurred
Figure 4. Simultaneous recordings of giant fiber spike conduction across the grafted region. A) Two LGF spikes, evoked by light tactile stimulation to one end of the preparation, were conducted across the grafted region (vertical bar) and into the other half of the preparation. B) The MGF spike, evoked by just-threshold electrical stimulation (arrow) through the body wall, was conducted across the grafted region and into the other half of the preparation.
only in an anterior direction in three animals and in a posterior direction in one animal. In posterior transections, the LGF initially conducted only in an anterior direction in three animals and only in a posterior direction in two animals. In all cases, of initially unidirectional conduction, spike conduction across the transection became bidirectional within 4-8 h.

In eleven of 13 grafted preparations, through-conduction of the LGF initially was bidirectional. In the other two preparations, LGF conduction was initially unidirectional but became bidirectional after one to two days. In preparations exhibiting MGF through-conduction (n = 6), the conduction was unidirectional in two animals but became bidirectional after two days.

One pattern of electrical activity seen in grafted preparations, but not in worms with transected VNCs, was a rebounding of giant fiber spikes (Fig. 5), similar to that observed during early stages of giant fiber regeneration following VNC transection in *Lumbricus terrestris* (Balter et al., 1980). Rebounding in grafted preparations occurred in four of 13 reconnected LGFs and in one of six reconnected MGFs. In general, rebounding was seen for only a few days after through-conduction was established.

Conduction Times Across the Lesion

One characteristic of regenerating giant fibers was the relatively long time required for spike conduction across the transection. To quantify this parameter, conduction times were measured over a 2 mm
Figure 5. Lateral giant fiber rebound spike in a grafted preparation
A pair of LGF spikes (lower four traces), evoked by tactile
stimulation, were conducted toward the graft site (bar). The first
spike failed, but the second spike was conducted across the graft. The
LGF rebound spike (dot) was initiated at approximately the same time as
the single spike on the other side of the graft. The rebound spike
then propagated away from the graft site as indicated by its inverted
waveform.
distance which included the transection site. No significant differences were found at anterior versus posterior sites in MGF and LGF conduction times and therefore these times were combined. Initially, the conduction time for MGF spikes across the transection was 2.4 ms + 0.2 SE (n = 5), corresponding to a conduction velocity of 0.8 m/s (Fig. 6A). In comparison, MGF velocity 1-3 mm away from the transection site was 7.2 m/s + 0.1 SE (n = 5) or nearly identical to that seen in the normal MGF.

Initially, the conduction time for LGF spikes across the transection was 1.1 ms + 0.1 SE (n = 5), corresponding to a velocity of 1.8 m/s (Fig. 6A). In comparison, LGF velocity 1-3 mm away from the transection site was 5.1 m/s + 0.1 SE (n = 5) or nearly identical to that seen in the normal LGF.

In all of the grafted preparations, there was also a long conduction time for the giant fiber spikes across the lesion. Conduction times were measured using a method similar to that in animals with transected VNC. Initially, for the six through-conducting MGFs the conduction time was 2.4 ms ± 0.2 SE (n = 6), corresponding to a velocity of 0.8 m/s (Fig. 6B). In comparison, MGF velocity 2-4 mm away from the grafted site was 2.6 m/s ± 0.2 SE (Fig. 6B). Initially, based on measurements in six of 13 preparations LGF conduction time for LGF spikes across the grafted region was 1.7 ms ± 0.1 SE, corresponding to a velocity of 0.7 m/s (Fig. 6B). In comparison, LGF velocity 1-3 mm from the grafted site was 2.7 m/s ± 0.1 SE and at 3-5 mm from the graft was 3.9 m/s ± 0.1 SE.
Figure 6. Initial conduction times across and adjacent to the transection or graft. A) MGF and LGF conduction times (mean ± SE, n = 5) across the transection were much greater than in adjacent anterior or posterior segments. B) MGF and LGF conduction times (mean ± SE, n = 6) across the graft site were also much greater than in adjacent segments. Data for the LGF were not necessarily from the same preparations as the MGF. Large stimulus artifacts prevented measurements of MGF conduction time at 5 mm from the grafted site on one end of the preparation. The dotted lines represent conduction times over 2 mm distance in normal (unoperated) animals.
A

CONDUCTION TIME (ms)

MGF

LGF

DISTANCE FROM TRANSECTION* (mm)

B

CONDUCTION TIME (ms)

MGF

LGF

DISTANCE FROM GRAFT* (mm)
Figure 7. Giant fiber conduction times across the transection at various times after initial through-conduction. Measurements were taken over a 2 mm distance which spanned the transected region and all points represent pooled data from anterior and posterior transection sites. MGF (solid circles) and LGF (open circles) conduction times (mean ± SE, n = 5) steadily decreased and by 100 h were essentially normal.
Gradually conduction times across the transection decreased for both giant fibers (Fig. 7). By 24 h after initial through-conduction, the conduction times across the transection for MGF and LGF had decreased to 0.8 ms ± 0.1 SE (n = 5) and 0.7 ms ± 0.1 SE (n = 5), respectively. By 100 h, conduction times across the transection were nearly normal.

In grafted preparations, decreases in conduction times across the grafted region were also evident (Fig. 8). By three days, conduction time across this region for the MGF and LGF had decreased to 2.2 ms ± 0.2 SE (n = 6) and 1.5 ms ± 0.1 SE (n = 13), respectively. By 14 days, conduction time for the MGF was 1.6 ms ± 0.2 SE and the LGF 1.1 ms ± 0.1 SE. By 21 days, conduction time across the graft for the LGF was 1.1 ms ± 0.1 SE and the MGF 1.4 ms ± 0.35 SE (n = 3). Even though grafted preparations were unable to obtain food and lost weight, they survived and giant fiber connections remained functional for 6-8 weeks.

Regeneration and Lability of Conduction in Posterior Segments

Recovery of MGF and LGF through-conduction in preparations consisting of the last 40 segments of worms occurred in 37 h ± 10 SD and 39 h ± 6 SD (n = 14), respectively. Although slightly slower, these times were not statistically different (Student t-test) from the recovery of giant fiber through-conduction in whole animals with posterior transections (40 h ± 11 SD for the MGF and 46 h ± 14 SD for the LGF, n = 20).
Figure 8. Giant fiber conduction times across the graft after initial through-conduction. All conduction times were measured over a 2 mm distance which spanned the grafted region. MGF (solid circles) and LGF (open circles) conduction times (mean ± SE, n = 6 for MGF, n = 13 for LGF) gradually decreased, but after two weeks were still greater than normal. The mean MGF velocity after 21 days (*) was based on three animals.
DAYS AFTER THROUGH-CONDUCTION

CONDUCTION TIME (ms)

* n=3
Recovery of spike conduction across transections in these preparations was similar to that in whole animals. Initially, conduction of single LGF spikes was bidirectional but occasionally intermittent. Bidirectionality of MGF spikes could not be tested because selective electrical stimulation of MGF, in the absence of LGF activity, was seldom possible. Also, as in whole worms (Fig. 7), conduction times across the transection in posterior segments gradually decreased. After the first 24 h following establishment of reliable through-conduction, the conduction time for the MGF decreased from 2.1 ms to 1.2 ms and the LGF from 1.2 ms to 1.0 ms. Conduction time continued to decrease over the next few days and was essentially normal by 96 h after transection.

Before testing the lability of conduction in these preparations, initial values of MGF conduction times across the transection were obtained. These times were then plotted against the time to through-conduction failure during 20 Hz stimulation (Fig. 9). After the first few seconds of stimulation, MGF-mediated muscle potentials decreased in amplitude and within approximately 30 s were not detectable along the entire preparation. Within the next minute, MGF-mediated motor neuron spikes were no longer detected at most recording sites. During this time, MGF conduction across the transection was reliable but the conduction time increased to 3-4 ms. Finally, after further stimulation, conduction of MGF spikes failed across the transection. As shown in Figure 9, conduction across the transection failed sooner in
Figure 9. Relationship between MGF conduction time across the transection and time to failure of through-conduction during 20 Hz stimulation. Each point represents a single preparation (posterior 40 segments of a worm). Preparations with the longest conduction times failed sooner than preparations with shorter conduction times.
preparations in which initial conduction times were the greatest (i.e., 2.0 ms or more).

Recovery of MGF through-conduction following stimulus-induced failure occurred within 20-30 min; immediately thereafter conduction times across the transection were approximately 1-2 ms greater than before stimulation. However, within 30 min conduction time through the transection returned to values seen before 20 Hz stimulation (Fig. 10).

Morphology

Single VNC Transection

Cross-sections of the VNC were studied in three animals sacrificed 4, 12, and 24 h after recovery of giant fiber through-conduction (i.e., 24, 38, and 47 h after cutting the VNC). In all these animals, there was no apparent giant fiber degeneration and the severed ends of the VNC were connected by an isthmus of tissue (approximately 0.5 mm long). At distances greater than 100 μm from the narrowest (middle) portion of the isthmus, the VNC appeared normal (Fig. 11A); that is, three dorsal giant fiber profiles, central neuropile, and large ventral cell bodies were evident in each section. At approximately 50-100 μm from the middle of the isthmus 4-5 giant fiber profiles, rather than three, were evident in two of three animals examined. Serial section analysis indicated these "extra" profiles were branch-like extensions originating from the giant fibers.

No large diameter profiles were evident in the middle of the isthmus. Instead numerous (5-10) profiles, each measuring 5-10 μm in
Figure 10. Changes in MGF conduction time across the transection following stimulus-induced failure of conduction. Points represent means for three individual preparations. Conduction times markedly decreased during the first minute and by 10 min were nearly the same as before stimulus-induced failure.
Figure 11. Cross-sections of VNC in lesioned animals  
A) Approximately 1 mm from the transected site (47 h after cutting), the VNC of an animal appeared normal with dorsal giant fiber profiles and centrally located neuropile.  
B) No large diameter giant fiber profiles were evident at the transection site in the same animal. One profile (arrow) was traced via serial sections to the MGF on both sides of the region.  
C) In a grafted animal, the VNC appeared nearly normal 2 mm from the graft site 72 h after MGF through-conduction. Note the close apposition of the VNC to the body wall in comparison to (A).  
D) Three connecting MGF and LGF in adjacent segments. A mat of dense tissue surrounded the VNC in the grafted region.
diameter were seen (Fig. 11B). Most of these profiles could be traced via serial sections to giant fiber origins, both anterior and posterior to the isthmus. In a few instances, profiles within the transected area were too small to be traced. As shown in Figure 11B, a thick mat of tissue surrounded the VNC. This mat was not seen around the VNC in unlesioned animals or in regions adjacent to the isthmus.

The isthmus region was studied in two animals at 72 and 96 h after lesioning. At this time, only three to six giant fiber profiles (10-15 μm diameter) were evident. The apparent reduction in the total number of profiles, as well as the general increase in profile diameter, suggested either a fusion or a selective enlargement of the giant fiber extensions.

**Grafted Preparations**

Cross-sections of VNC were obtained in three grafted preparations approximately 72 h after recovery of both LGF and MGF through-conduction. An isthmus of tissue (approximately 3-5 mm long) connected the two ends of the VNC from both animals. At distances greater than 250 μm from the middle of the isthmus, the VNC appeared normal (i.e., three dorsal giant fiber profiles, central neuropile, and ventral cell bodies). As in the case of the transected VNC, the isthmus was enveloped by a thick mat of tissue.

In the middle of the isthmus, three conspicuous profiles (Fig. 11D), approximately 15-20 μm in diameter were evident in all three preparations. In all cases, these profiles were traceable in both
directions to the giant fibers in adjacent segments. In two preparations, one to two additional small diameter profiles (5-10 μm) were seen in the isthmus. Serial section analysis indicated these were branch-like extensions of the giant fibers.
DISCUSSION

Regeneration of giant fibers in *Eisenia foetida* occurred in less than 24 h, but usually by 38-48 h following VNC transection. By comparison, in *Lumbricus terrestris*, through-conduction of regenerating giant fibers occurred five to twelve days following VNC transection (Balter et al., 1980). Two factors which most likely contributed to the faster recovery rate in *Eisenia foetida* were the smaller distance between the cut ends of the regenerating giant fibers (approximately 0.5 mm in *Eisenia foetida* versus 1.0 mm in *Lumbricus terrestris*) and the higher maintenance temperature (27°C) for *Eisenia foetida* versus 15°C for *Lumbricus terrestris* (Balter et al., 1980).

In the MGF and LGF, there were no apparent differences in the time to recovery of through-conduction at anterior versus posterior transection sites. These results suggest that the various gradients in sensory and motor properties of the giant fibers do not significantly influence the recovery of giant fiber through-conduction. Also, results from posterior portions of worms consisting of approximately the last 40 segments indicated that the recovery of spike through-conduction was not altered by the absence of the head.

Physiological results and serial section analysis from animals with transected VNCs indicated that MGF and LGF regeneration in *Eisenia foetida* was always specific. Such specificity has also been reported in *Lumbricus terrestris* following VNC transection (Birse and Bittner, 1976; Balter et al., 1980; Birse and Bittner, 1981). Thus, specificity of
giant fiber reconnections seems to be a general feature of earthworm central nervous system regeneration.

The time required for establishing giant fiber through-conduction in grafted animals was considerably longer than in either whole animals or posterior portions of animals with single VNC transections. Two obvious factors possibly contributing to this difference were the relatively longer distance between the cut ends of the grafted VNCs and the unavoidable disruption of blood circulation in the vicinity of the graft. Other factors which may have contributed to this difference were possible tissue rejection or reduced effectiveness of target cell recognition between giant fibers in the grafted ends. An immune response, consisting of a cellular component and possible humoral component, has been reported in numerous species of earthworms (Cooper, 1979). For example, *Eisenia fetida* body wall grafts were partially rejected after several days (Cooper and Rubilotta, 1969). Apparently, any rejection response in grafted preparations in the present study was not sufficiently strong to prevent specific and long-term connections between the LGFs. On the other hand, MGF through-conduction was evident in only 50% of the grafted preparations. This may indicate either a reduced effectiveness of target cell recognition or rejection.

**Spike Conduction in Regenerating Giant Fibers**

Conduction properties in regenerating giant fibers following VNC transection were similar to those in grafted animals in several ways. First, conduction of giant fiber spikes across the lesion was initially
intermittent and labile. Second, giant fiber spike conduction in both transected and grafted preparations was slow across the lesion (Figs. 2, 3, and 4). Third, slow conduction was limited to a relatively small area on either side of the lesion (Fig. 6). These properties most likely reflect the small diameter of the giant fibers in the lesioned area and may be indicative of an associated reduction in the safety factor for spike conduction. These properties are also consistent with previously proposed models of electrical coupling between the severed ends of the fibers (Birse and Bittner, 1976; Balter et al., 1980; Birse and Bittner, 1981).

In both transected and grafted preparations, through-conduction of spikes became more reliable and conduction time across the lesion decreased (Figs. 7 and 8). Corresponding to these changes, giant fiber profiles in the transected area at later stages appeared less numerous and larger; however, it was not clear whether such changes involved selective fusion, growth or retraction of the giant fiber extensions. Also, it was not clear why MGF conduction times were consistently greater than those of the LGF after establishment of through-conduction in transected and grafted preparations.

The results from the present study indicated spike propagation across newly regenerated giant fibers failed after repetitive stimulation and that this failure was reversible (Figs. 9 and 10). Assuming regeneration of giant fibers was the result of direct fusion or establishment of an electrical synapse between severed axons, then one
possibility for conduction failure was that repetitive stimulation somehow decreased the electrical coupling between the regenerating axons. An alternative explanation was that repetitive stimulation caused a gradual reduction in the safety factor for giant fiber spike conduction. Previous studies in polychaete and oligochaete giant fibers (Bullock and Turner, 1950; Bullock, 1953; Mellon et al., 1980), cockroach giant fibers (Parnas et al., 1969), and crayfish motor neurons (Parnas, 1972; Smith, 1980) have demonstrated failure of spike propagation during repetitive stimulation at narrow regions or branch points along the fibers. In these examples, failure typically involved increased conduction times during repetitive stimulation, followed by failure, and eventual recovery.

The establishment of giant fiber cross-connections in grafted preparations raises the possibility of studying these connections in more unusual grafts, such as those involving union of rotated worms or union between different species (Joest, 1897; Morgan, 1897; Hazen, 1899; Morgan, 1901; Korschelt, 1931). Such manipulations, in combination with current electrophysiological and morphological techniques, may be useful in studying processes by which neurons find their appropriate targets during development or regeneration.
SUMMARY

Rapid and specific reconnection of giant nerve fibers occurred following single ventral nerve cord (VNC) transection or grafting in the earthworm, Eisenia fetida. Through-conduction of medial (MGF) and lateral (LGF) giant fiber spikes was seen as early as 20 h (n = 5), but usually between 38-48 h (n = 35) after transection. No differences were detected in the time to through-conduction at anterior versus posterior transection sites, but MGF through-conduction usually preceded that of the LGF. Spike conduction across the lesion was initially intermittent and labile but became more reliable in 4-8 h. Rapid and specific reconnection of the giant nerve fibers also occurred in preparations consisting of 40 posterior segments. In these preparations, a reversible failure of MGF spike through-conduction was demonstrated with repetitive stimulation (20 Hz).

Through-conduction of LGF and MGF spikes in grafted preparations occurred after 65 h ± 26 SD (n = 13) and 7-14 days (n = 6), respectively. Conduction properties of MGF and LGF spikes in grafted preparations included bidirectional conduction, delayed conduction across the graft, and rebounding. Grafted preparations survived at least 8-10 weeks.

Conduction properties of regenerating giant fibers in transected and grafted preparations were consistent with the morphological properties of the lesioned area. These properties included the directed outgrowth of numerous, small diameter profiles which emerged from the
cut ends of the giant fibers. Physiological and morphological evidence was also consistent with the current hypothesis that regeneration of earthworm giant fibers occurred by direct electrical interaction involving either the formation of an electrical synapse or direct fusion.
REFERENCES


PART II. VENTRAL NERVE CORD TRANSPLANTATION IN THE EARTHWORM, EISENIA FOETIDA. 1. MORPHOLOGY
INTRODUCTION

Neural transplantation is a useful approach in understanding basic mechanisms of nervous system development. In particular, questions pertaining to the developmental plasticity of transplanted tissue and to the interactions of transplanted and host tissue can be addressed. Within the context of neural transplantation therapy, questions arise pertaining to transplantation related compensation for genetic, developmental, or injury related deficits.

In higher vertebrates there have been extensive studies involving central nervous system transplantation (Wallace and Das, 1983). The success of these transplants is generally based on the morphological integrity of the tissue following surgery (Das, 1983). Due to the complexity of vertebrate central nervous systems, there have been few electrophysiological studies of the connectivity of transplanted neurons with host neural tissue. Three noteworthy studies demonstrated that electrical stimulation of the transplanted tissues (e.g., septal nucleus, locus coeruleus, or optic tectum) altered electrical activity in the recipient nervous system, whereas stimulation of the surrounding tissue did not alter the electrical activity in the recipient nervous system, indicating a functional interaction between transplanted tissue and the recipient nervous system (Bjorklund et al., 1979; Harvey et al., 1982; Low et al., 1982). In most neural transplantation studies, electrophysiological testing has not been done and functional interactions between the transplant and recipient tissue have been
inferred from behavioral observations (Perlow, 1980; Hallas, 1982; Wallace and Das, 1982; Dunnett et al., 1983; Labbe et al., 1983; Perlow, 1983; Wallace, 1983).

Surprisingly, in spite of the presence of large identifiable neurons and well developed capabilities for regeneration, there have been relatively few studies of neural transplantation in invertebrates. In most of these studies whole ganglia or whole ganglia plus target tissues were excised from donor animals and transplanted to ectopic locations. For example, in molluscs, cells in transplanted ganglia and associated target tissue survived and formed a variety of appropriate as well as novel connections with other cells of the transplanted ganglionic or target tissue (Murphey and Kater, 1978; Murphey and Kater, 1980; Bulloch and Kater, 1982). In other studies, connections were established between transplanted neural tissue and peripheral target tissue of the recipient (Bodenstein, 1957; Guthrie, 1966; Jacklet and Cohen, 1967; Guthrie and Banks, 1969). Studies by Zhang and Nicholls (1983) have demonstrated that single neurons implanted onto whole ganglia survived, formed sprouts, and made functional connections with appropriate target neurons. Additional studies have demonstrated the growth of nerve fibers between grafted appendages (e.g., legs or cerci) and the recipient nervous system (Edwards and Sahota, 1976; Young, 1972; Palka and Schubiger, 1975; Murphey et al., 1981) although in at least one case the growing neurons did not form functional connections with their proper target neurons (Murphey et al., 1983).
There have been only a few invertebrate studies involving excision of neural tissue from one animal and replacement with the corresponding tissue from another animal. For example, Page (1982) transplanted optic lobes between cockroaches raised in different light cycles and found a transfer of circadian activity rhythm. In another study, Schneiderman et al. (1982) found transplanted male antenna imaginal disk retained its responsiveness to pheremone stimulation after transplantation to female larvae in *Manduca sexta*.

One objective of the present study was to determine if lengths of ventral nerve cord (VNC) transplanted into denervated regions of VNC in another animal would survive and form functional connections, both centrally and peripherally, with the recipient. In particular, special attention was given to the morphological changes of the giant nerve fibers in the transplanted tissue and to probable interconnections between transplanted and recipient giant fibers. Such interconnections might be expected in view of the syncytial organization of the giant fibers (Gunther, 1975) and the high capacity for regeneration of the giant fibers (Birse and Bittner, 1976; Balter et al., 1980; Birse and Bittner, 1981; Part I).

Another objective was to determine if central connections would be altered after transplantation of VNC to a different location (anterior versus posterior). This might be expected in view of physiological evidence for longitudinal gradients of the giant fibers with respect to sensory inputs (Gunther, 1973; Moore, 1979) and motor outputs (Drewes and McFall, 1980; Drewes et al., 1980; O'Gara et al., 1982).
MATERIALS AND METHODS
Transplantation Procedure

Adult earthworms, *Eisenia fetida*, were obtained from a laboratory culture (O'Gara et al., 1982). A donor animal was anesthetized on ice for approximately one minute and pinned ventral side up, through the lateral margins of the body wall, to a sheet of cold dental wax. To expose the ventral nerve cord (VNC) a longitudinal incision approximately 15 segments long was made through the ventral midline of the body wall. Twelve segments of VNC were removed (donor VNC) and placed into worm saline (Drewes and Pax, 1974). The recipient worm was then cooled on ice, pinned ventral side up, and twelve segments of its VNC were removed and discarded. The donor VNC was then placed lengthwise into the denervated segments of the recipient animal. Care was taken not to puncture the gut or the ventral blood vessel. The body wall was sewn together with three to four stitches using 6-0 silk glued to a bent minuten pin. The animal was placed into a petri dish containing moist dirt and manure (27°C).

Animals were divided into two groups based on the segmental origins of donor and recipient VNC. In Group I a posterior portion of VNC (segments 75-87) in the recipient worm was replaced with VNC segments 75-87 from a donor worm (there are approximately 100 segments in *Eisenia fetida*). In Group II VNC segments 75-87 were replaced with an anterior portion of the VNC (segment 10-22) from a donor. Approximately 30% of
the transplanted VNC formed functional connections with the recipient VNC and body wall (Part III).

Histology

Histological studies of animals from Groups I and II were made 1) before recovery of giant fiber through-conduction (two to four days after transplantation), 2) within 24 h after establishment of through-conduction in both giant fibers (four to eight days after transplantation), 3) four to five weeks after transplantation, and 4) nine to ten months after transplantation. Animals were anesthetized on ice, pinned ventral side down to cold dental wax, and dissected to expose the VNC in the transplant region or denervated region. The VNC from segments 73-89 along with attached body wall were removed and pinned to a thin slab of Sylgard and immersed overnight in 2.5% glutaraldehyde in 0.1 M cacodylate buffer at 5°C. Following fixation the excess lateral edges of body wall were trimmed away leaving a thin strip of body wall approximately 3-4 mm wide along with transplanted VNC. This strip was then pinned to a Sylgard slab, dehydrated in a graded series of ethanol, cleared in two changes of xylene, and embedded in paraffin. Serial cross sections, 5 μm thick, were cut and stained with 0.5% toluidine blue in 0.5% borax.
RESULTS

Gross Morphology of the Transplant Region

By 24 h after transplantation, the incision along the ventral body wall had healed and appeared externally as a thin white line. For several weeks after surgery, the body diameter throughout the transplant region was noticeably smaller than in adjacent unoperated segments, but within four to six weeks the diameter was nearly normal. Another characteristic throughout the transplant region was a dark pigmentation of the body wall. This darkening developed within a few days after surgery and persisted for at least ten months.

By 48 h after surgery, the transplanted VNC in freshly dissected worms was completely covered by a thick, dense mat of white tissue. The density of this mat was greatest after four to seven days and then gradually decreased over the next several weeks, but usually did not disappear entirely. No segmental nerves or segmental blood vessels were discernable through the mat, even after the thickness of the mat had diminished. In all cases, the ventral blood vessel was absent in the transplant region, although it had been left intact during transplantation. However, there was a diffuse network of fine blood vessels penetrating the mat and often a pair of longitudinal vessels lateral to the transplanted VNC.

Morphology Prior to Giant Fiber Through-conduction

By two days after transplantation (Groups I and II), there was a bud-like formation, approximately 150-210 μm in length extending from
each cut end of the donor and recipient VNC (Fig. 1A, 1B, and 1C). The organization and staining in most of the bud somewhat resembled the neuropile of adjacent VNC. A gap of approximately 200-400 \( \mu m \) separated the opposing bud-like formation on each end of the donor and recipient VNC (Fig. 1D).

At two days, several nerve fiber profiles (8-25 \( \mu m \)) were evident throughout most of the length of the VNC buds and, based on serial section analysis, the profiles originated from the MGF and LGF in adjacent VNC. The diameter of these profiles was smallest near the tip of the bud and largest adjacent to the VNC.

By four days, a 80-140 \( \mu m \) gap separated the buds of the donor and recipient VNC. It was not clear whether the reduction in the gap was entirely due to directed growth of the bud-like formations or to a general drawing together of body wall tissue between donor and recipient VNC.

**Morphology After Giant Fiber Through-conduction**

Serial sections of transplanted and adjacent VNC were studied in Groups I and II at three times after establishment of giant fiber through-conduction: 1) within 24 h after establishment of through-conduction in both giant fibers (usually four to eight days after transplantation, Part III), 2) four to five weeks after transplantation, and 3) nine to ten months after transplantation. Special attention was given to the junction connecting the donor and recipient VNC and to morphological changes within the transplanted VNC.
Figure 1. Cross-sections of the transplant region two days after surgery. 
A) Throughout most of its length the transplanted VNC appeared normal with three dorsal giant fibers and central neuropile. 
B) At 95 μm from the end of the transplanted VNC, the VNC was reduced in diameter and covered with a dense mat of tissue. Note the dorsal MGF (M) and LGF (L) profiles. 
C) At the very tip of the transplanted VNC a small bud-like extension was evident. 
D) In the gap between donor and recipient VNC, no neural tissue was evident. 
Abbreviations: cc-coelomic cavity; Calibration bar: A,B,D-100 μm; C-64 μm.
Junction

At initial establishment of giant fiber through-conduction, the bud-like formations from the donor and recipient were fused to one another. Although the morphology of the giant fibers in the junctional region between recipient and donor VNC varied depending on the specific orientation and alignment of the transplanted VNC with respect to the recipient VNC, the junctional configuration did not appear altered by the anterior-posterior orientation of the transplanted VNC, by rotation around the longitudinal axis of the transplanted VNC, or by time (4-5 weeks and 9-10 months) after transplantation.

In approximately two-thirds of the junctions in Groups I and II, the transplanted and recipient VNC were slightly displaced in the dorsal-ventral direction, creating an over-under (i.e., stacked) appearance. In these cases, donor and recipient giant fibers were directly united, in parallel to one another, by unusually large diameter connections (Figs. 2 and 3). The diameters of the giant fibers within this region were 40-62 μm as compared to 10-30 μm in normal worms.

In the remaining one-third of the junctions from Groups I and II, the transplant and recipient VNC were slightly displaced in a transverse direction (i.e., side-by-side). In these cases, there were typically more than three giant fiber profiles (10-25 μm) in the junction (Fig. 4).
Figure 2. Cross-sections of VNC from the junctional, normal (recipient), and transplant (donor) regions seven days after surgery in a Group II animal. In the junctional region, the MGF and LGF profiles from the recipient VNC, (shown in 5 μm serial sections) united in parallel with the corresponding giant fibers from the transplanted VNC. Note, in this example, the reduced cross-sectional area of neuropile in the region of giant fiber union. Throughout most of its length, the transplanted VNC was completely surrounded by a dense mat of tissue. Calibration bar: junction-100 μm, normal and transplant-90 μm.
Figure 3. Three dimensional reconstruction of the united giant fibers in the junctional region (same animal as is Fig. 2). The upper portions of the giant fibers arise from recipient VNC and the lower portions from the donor VNC.
Figure 4. Serial cross-sections of the junctional region five weeks after surgery. A) The transplanted VNC (left) was fused with the recipient VNC (right). Three dorsal giant fibers (L-LGF, M-MGF) were evident in the recipient VNC. Numerous profiles were seen in the donor (i.e., transplanted) VNC, one of which (arrow) united with the LGF from the recipient nerve cord in (B) and (C). Note the relatively thin tissue mat overlying the VNC and the close apposition of VNC to the body wall.
**Transplant**

After giant fiber through-conduction was first established in the transplant, the cross-sectional appearance of the VNC in the transplant region appeared relatively normal as indicated by the presence of one MGF, two LGFs, central neuropile, and numerous large ventral cell bodies. One noticeable difference was a slight thickening of the sheath surrounding each giant fiber. Also, there was little or no space between the transplanted VNC and the body wall.

In cross-sections at four to five weeks after transplantation, numerous profiles (up to 16) were seen in close proximity to the giant fibers in the transplanted VNC in both Groups I and II (Fig. 5A and 5B). Serial section analysis indicated virtually all of these profiles were branch-like extensions from either MGF or LGF. Typically, such branches arose approximately every 100 μm along the giant fibers and were 10-15 μm in diameter, 50-60 μm long, and ended blindly near the giant fibers.

To test whether formation of these branches in the transplant may be related to some type of foreign tissue rejection (Cooper, 1979) or to inappropriate anterior-posterior orientation of the transplanted VNC, nerve cord segments 75-87 were removed and immediately implanted into the same animal in the denervated location. In three animals, implantation of the VNC was made with the correct anterior to posterior orientation and in three other worms the orientation was reversed. However, in all cases comparable branching of the giant fibers was seen after five weeks, indicating that neither foreign tissue rejection nor
Figure 5. Cross-sections of transplanted VNC. Numerous giant fiber profiles were evident in the transplant at four weeks (A) and five weeks (B) after surgery. In (B), unlike (A), the neuropile appeared abnormal, the VNC was closely apposed to the body wall, and there was no subneural blood vessel. C) In another preparation, nine months after transplantation two large profiles were detected in the center of the VNC, rather than dorsally, in some sections. The neuropile appeared abnormal and no cell bodies were evident in the ventral portion of the VNC. D) Ten microns from the section in (C) only one large profile was seen.
transplant orientation were critical factors contributing to the induction of profuse branching of giant fibers.

Nine to ten months following transplantation, further changes in transplanted VNC morphology were evident in both Groups I and II. Though multiple profiles were seen in many sections, in other sections only two large profiles (20-50 μm diameter) were evident (Fig. 5C), but these could not be serially traced to the giant fibers in the junctional region. The sheath could not be detected around the giant fiber profiles in many sections. In one animal only a single profile was seen in some sections (Fig. 5D).

Nine to ten months after transplantation in both Groups I and II, the cross-sectional area of the transplanted VNC in some sections was decreased to approximately 30-50% of that in adjacent normal segments. One factor contributing to this decrease was an apparent reduction in the number of large ventral cell bodies present in normal VNC. Another factor contributing to this decrease was a proportionate reduction in the cross-sectional area of neuropile and a nearly proportionate decrease in giant fiber cross-sectional area.

Peripheral Connections

Two days after transplantation, the original segmental nerve stumps were evident along most of the transplanted VNC. Four days after transplantation, segmental nerve stumps were still visible in some regions, but were generally less conspicuous. In both Groups I and II a few very fine processes emerged ventrally or laterally from the
Figure 6. Cross-sections of the transplant region showing the probable connections between transplanted VNC and body wall. A) Fine processes (white arrow) projected from the VNC into the body wall. B) A bundle of fibers emerging from the lateral edge of the transplanted VNC penetrated (arrows) into the body wall. In this animal the VNC was inadvertently transplanted up-side-down.
transplanted VNC and penetrated into the body wall (Fig. 6A). By seven days, more diffuse processes were seen projecting from the transplanted VNC into the body wall (Fig. 6A), but there was no obvious pattern of their projection along the length of the transplant. At 4-5 weeks and 9-10 months these processes were still present.

In addition to the diffuse processes, discrete bundles of fibers arising from the lateral margins of the transplanted VNC were seen at 4-5 weeks and at 9-10 months in both Groups I and II (Fig. 6B). These bundles were reminiscent of segmental nerves but were asymmetrically arranged and often wider and larger than segmental nerves in normal worms. In most instances, they penetrated directly into the body wall musculature.
DISCUSSION

Interconnections

Transplanted VNC united with the VNC in recipient animals by a junction involving directed outgrowths from the severed ends of the nerve cords. One week after transplantation the MGF and LGF in the junctional region appeared fused with their counterparts from the recipient VNC. Giant fibers showed no apparent septal partitions and their diameters appeared normal or greater than normal in the junctional region (Figs. 2, 3, and 4). The failure of giant fiber degeneration in transplanted and adjacent recipient VNCs is probably related to the multisegmental origin of these neurons (Mulloney, 1970; Gunther, 1971) and the limited extent of VNC degeneration following transection (Birse and Bittner, 1976; Birse and Bittner, 1981; Part I).

The mechanisms for nerve fiber regeneration are diverse in invertebrates (Guthrie, 1975). Some studies have demonstrated fusion of proximal and distal stumps of regenerating crustacean motor neurons (Hoy et al., 1967; Bittner and Johnson, 1974; Kennedy and Bittner, 1974) and leech sensory neurons (Deriemer et al., 1983). These fusions involved many small diameter sprouts growing from the cut ends of the neurons. Similarly, numerous small diameter giant fiber processes joined one another after VNC transection or grafting in earthworms. Establishment of these connections was closely associated with recovery of through-conduction of giant fiber spikes (Part I). In contrast, the initial joining of the giant fibers in transplanted VNCs involved
relatively large diameter connections, whose formation was associated with recovery of giant fiber through-conduction (Part II). Thus, it appears that the determinants of giant fiber radial growth and the requisite anatomical configuration for giant fiber through-conduction in the regenerating region of the transplanted VNC may be different than in VNC transection or grafting.

The anatomical configuration of the giant fiber connections in the junctional region was stable for many months and appeared independent of foreignness, anterior-posterior orientation, or rotational orientation of the transplanted VNC. In cases where the orientation of the transplanted VNC was reversed (anterior-posterior) or inadvertently rotated (upside-down), the left LGF of the transplanted VNC united with the right LGF of the recipient VNC and vice-versa. Thus, it appears that regenerating giant fibers can recognize and reconnect with the appropriate target (LGF versus MGF) but there appears to be no lateral specificity with respect to LGF connections. Birse and Bittner (1981) reported somewhat similar results in Lumbricus terrestris in which transected LGFs, although correctly oriented, occasionally formed connections with contralateral LGFs on the other side of the lesion.

In addition to the longitudinal interconnections between the transplanted and recipient VNC, peripheral connections were established between the body wall of the recipient animal and the transplanted VNC. The number and configuration of these connections appeared to be independent of the origin of the transplanted VNC. These connections occasionally involved the original segmental nerves but often involved
very fine processes which projected along new, ventrally oriented pathways between the VNC and the body wall musculature (Fig. 6). It seems reasonable to assume that these connections might be regenerating fibers originating from either central or peripheral neurons. Physiological evidence that formation of these connections is accompanied by restoration of motor and sensory function in the transplant region is presented in Part III.

Morphological Stability

In contrast to the long-term stability of the giant fiber morphology in the junctional region, considerable morphological changes were evident in all transplanted VNC, irrespective of origin. By four to five weeks, the giant fibers had formed numerous branch-like extensions. By nine to ten months, these extensions were reduced in number or absent and the neuropile was often reduced in cross-sectional area. As indicated by the results, the critical factor responsible for these changes appeared to be the severing of peripheral nerves and associated blood vessels, rather than foreign tissue rejection or an inadvertent anterior-posterior reversal of the transplanted VNC.

The severing of peripheral nerves necessarily involves disruption of sensory inputs and motor outputs of the giant fibers. This disruption, along with possible failure of these pathways to regenerate, may have triggered a cascading or compensatory disruption in the morphological integrity of the VNC, including giant fiber organization. Other studies have shown that the loss of presynaptic neurons (via
transection of peripheral axons or ablation of sensory organs) can alter the dendritic morphology of the giant interneurons of crickets (Palka and Edwards, 1974; Matsumoto and Murphey, 1978; Murphey and Levine, 1980) and grasshopper embryos (Shankland et al., 1982). Comparable changes in dendritic morphology of postsynaptic neurons following disruption of afferent pathways has also been demonstrated in cockroach motor neurons (Pitman and Rand, 1982) and in vertebrate interneurons (Jones and Thomas, 1962, Globus and Scheibel, 1966; Globus and Scheibel, 1967; Rakic, 1975; Kimmel et al., 1977).

In summary, cell-specific giant fiber fusion occurred between transplanted and recipient giant fibers. In addition, peripheral connections were made between transplanted VNCs and recipient body wall. The following paper (Part III) presents evidence for the physiological integrity of these central and peripheral connections.
SUMMARY

Twelve segments of ventral nerve cord (VNC) from adult earthworm (*Eisenia fetida*) were transplanted into recipient worms from which a corresponding length of VNC had been removed. The transplants survived and formed anatomical connections with the VNC and body wall of the recipient animal. Irrespective of the orientation of the transplanted VNC, medial (MGF) and lateral (LGF) giant fibers connected to their counterparts in the recipient VNC. However, reconnection of the LGFs was not always laterally specific (e.g., in rotated transplants left LGFs connected to right LGFs). The configuration of these connections was stable for at least nine to ten months after transplantation. In contrast, the morphology of the giant fibers within middle portions of the transplanted VNC was less stable. Four to five weeks after transplantation, numerous branch-like processes extended from the giant fibers, but these were reduced or absent after nine to ten months. These changes were accompanied by other alterations in the morphology of the transplanted VNC, including a reduction in the number of ventral cell bodies, a slight reduction in cross-sectional area, and altered neuropile organization.
REFERENCES


PART III. VENTRAL NERVE CORD TRANSPLANTATION IN THE EARTHWORM, EISENIA FOETIDA. II. ELECTROPHYSIOLOGY AND BEHAVIOR
INTRODUCTION

The nervous system of earthworms is composed of more than 100 segmentally arranged ganglia containing serially homologous and identifiable neurons. Although there may be few, if any, external anatomical differences between segments in different body regions, there are pronounced intersegmental gradients and discontinuities with respect to nervous system design and function. These are especially evident in the neural circuitry underlying the giant fiber-mediated escape reflexes (Mill, 1975; Drewes, 1984).

Many of the neuronal constituents of the earthworm escape reflex circuitry have been identified and include touch and pressure sensory neurons (Gunther, 1970; Gunther, 1971a; Gunther, 1973), medial (MGF) and lateral (LGF) giant nerve fibers (Mulloney, 1970; Gunther, 1971b), and giant motor neurons (Gunther, 1972). The cell bodies of the touch and pressure sensory neurons are centrally located with axons projecting ipsilaterally to the body wall via segmental nerves. Although in many segments these mechanosensory inputs produce at least subthreshold excitation in both the MGF and LGF (Smith and Mittenthal, 1980), there are clearcut intersegmental gradients with respect to the effectiveness of mechanosensory inputs in evoking giant fiber spikes (Gunther, 1973). The consequence of these gradients in the intact animal is that MGF and LGF sensory fields overlap slightly, MGF spikes being evoked by touch in
the anterior one-half of the animal and LGF spikes by touch in the posterior two-thirds (Drewes et al., 1978; Moore, 1979).

Intersegmental gradients also exist with respect to motor components in the escape reflex of earthworms. For example, each MGF spike triggers spikes in segmentally arranged giant motor neurons, which have axons that project contralaterally to longitudinal muscles (Gunther and Walther, 1971; Gunther, 1972; Drewes et al., 1980). During repetitive firing the MGF-mediated muscle potentials show marked facilitation in amplitude (Gunther, 1972). In anterior segments, this facilitation is especially pronounced, whereas in posterior segments there is little or no facilitation (Drewes et al., 1980).

In the present study, we used neural transplantation procedures to investigate possible functional plasticity in various neural components of earthworm escape reflexes. Morphological evidence from the previous study (Part II) demonstrated that transplanted ventral nerve cord in the earthworm, Eisenia foetida formed cell-specific connections with recipient nerve cord and body wall, but it was not established whether such connections were functional and to what degree giant fiber reflexes were re-established or altered following such procedures. The objectives of this study were to determine if 1) functional connections were made centrally between transplanted ventral nerve cord and recipient ventral nerve cord, 2) functional connections were made peripherally between the transplanted ventral nerve cord and recipient body wall, and 3) intersegmental gradients associated with the sensory
and motor function of the giant fibers were retained or altered after transplantation.
MATERIALS AND METHODS

Pretesting Procedure

Adult *Eisenia fetida* were selected from a laboratory culture. Prior to ventral nerve cord (VNC) transplantation, longitudinal gradients in the facilitation of MGF-mediated muscle potentials were determined non-invasively using a printed circuit board grid (O'Gara et al., 1982). To do this the MGF was electrically stimulated through the body wall at approximately segment 50 (middle of the worm). Twin pulse stimulation (10 ms delay, 7-10 volts, 0.025 ms duration) reliably evoked a pair of MGF spikes and associated muscle potentials. The conduction velocity and waveform of these electrically evoked MGF responses were essentially identical to those of tactile evoked responses previously described by O'Gara et al. (1982). Measurement of facilitation was made at individual recording sites by determining the ratio of the amplitude of the second MGF-mediated muscle potential to the amplitude of the first. Comparisons were then made between average ratios for anterior (segments 10-25) and posterior locations (segments 75-85).

Sensory fields for both MGF and LGF were also determined in each animal. The worm was placed ventral side down on the recording grid and lightly touched on each segment with the tip of a minuten pin attached to a non-conductive handheld probe. Boundaries and overlap regions of the MGF and LGF sensory fields were determined. A segment was considered as touch-sensitive if touch to any part of the dorsal half of the segment consistently evoked giant fiber spiking. Boundaries of the
fields differed by several segments from one animal to another, but for a given animal the boundaries were highly repeatable from day to day.

Following determination of sensory fields and muscle potential facilitation ratios, animals were placed into either a donor group or recipient group for transplantation. For posterior sites of transplantation, MGF sensory fields in the recipients extended no further than segment 55; that is, the posterior limit of the MGF field was at least 20 segments anterior to the beginning of the transplant site. For anterior sites of transplantation, LGF sensory fields in the recipients extended no further than segment 32; that is, the anterior limit of the LGF field was at least 10 segments posterior to the beginning of the transplant site.

Transplantation

Twelve segments of donor VNC were transplanted into recipient animals as described previously (Part II). In Group I, posterior VNC was replaced by posterior VNC from a donor (Table 1). In Group II, posterior VNC was replaced with anterior VNC. In Group III, 12 segments of VNC (segments 75-87) were removed and not replaced with any transplanted tissue. In Group IV, anterior VNC was replaced with anterior VNC. In Group V, anterior was replaced with posterior VNC. Success rates for these transplantations were based on recovery of giant fiber spike conduction through the transplanted VNC, as determined by non-invasive recordings.
<table>
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<tr>
<th>Group No.</th>
<th>Segmental origin of donor VNC</th>
<th>Segmental site of VNC transplant into recipient</th>
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<tr>
<td>I</td>
<td>75-87</td>
<td>75-87</td>
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<tr>
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<td>V</td>
<td>75-87</td>
<td>10-22</td>
<td>34</td>
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<tr>
<td>Recovery of giant fiber through-conduction no. (%)</td>
<td>Unsuccessful attempts</td>
<td></td>
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<td>-------------------------------------------------</td>
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<td></td>
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Electrophysiological Procedures

**Non-invasive Recordings**

Beginning 24 h after transplantation, giant fiber responses were tested non-invasively each day for two to three weeks and every few days thereafter for the next six months. Special attention was given to physiological recovery of the following: 1) conduction of giant fiber spikes through the transplanted VNC, 2) giant fiber sensory fields in the transplant region, and 3) facilitation of MGF-mediated muscle potentials in the transplant region. These determinations were made by placing the animal across six adjacent pairs of recording electrodes (2 mm spacing between adjacent pairs). Two of the electrode pairs were immediately anterior, two within, and two immediately posterior to the transplant region. Positioning of the electrodes relative to the transplant was facilitated by the obviously dark pigmentation of only those segments receiving transplanted VNC (Part II).

**Intracellular Recordings**

Animals were anesthetized on ice, dissected to expose the VNC, and covered with a thin layer of saline (Drewes and Pax, 1974). Hook electrodes placed under the VNC anterior to the transplant were used for twin-pulse electrical stimulation of the MGF. Excitatory postsynaptic potentials (EPSPs) in giant fibers were evoked by touching the lateral edges of the opened body wall with an electromechanically driven probe. This probe consisted of a pin glued to a wooden stick which was mounted
on an elliptical speaker (2" X 6") connected to a Grass 44 stimulator. The maximal displacement of the pin was 0.5 mm.

Identically dissected preparations were used for intracellular verification of the functional recovery of MGF-mediated muscle potentials in the transplanted region. Facilitation of intracellularly recorded muscle potentials was then compared to facilitation in non-invasive recordings.
RESULTS

Recovery of Giant Fiber Through-conduction

Immediately after transplantation LGF and MGF spikes, evoked by tactile stimulation to the posterior and anterior ends of the animal, were reliably propagated toward and detected at recording sites immediately adjacent to the transplant. However, these spikes were not detected at either of the two sites within the transplant (Figs. 1A and 2A).

Recovery of giant fiber through-conduction (i.e., conduction into, through, and out of the transplant) was seen in a few animals as early as four days but usually between 6-10 days. In Group I, the mean recovery time for LGF through-conduction was 7.2 days ± 2.0 SD and for the MGF 9.2 days ± 2.8 SD (n = 6). In Group II, the mean recovery time for LGF was 5.9 ± 2.6 SD and for MGF was 7.9 days ± 0.8 SD (n = 8). In Group III (n = 6), no recovery of MGF or LGF occurred by seven weeks. In Group IV, recovery of MGF and LGF through-conduction occurred from 5-12 days after transplantation. In Group V, MGF recovery occurred from 5-8 days and LGF from 6-14 days after transplantation.

Lateral giant fiber spikes in transplanted VNC had a biphasic waveform, comparable to LGF spikes in adjacent and normal VNC. Initially, in a few animals, the LGF spike amplitude and duration in the transplant region were approximately 40 μV and 1.7 ms, or almost twice those of LGF spikes in normal or adjacent VNC. However, one week after
Figure 1. Non-invasive recordings of MGF responses to light tactile stimulation of the head in animals with transplanted VNC. A) Two days after transplantation in a Group II animal, a MGF spike (dot) conducted up to, but not into, the transplant region (vertical bar). Each MGF spike was followed after 1.2 ms by an apparent motor neuron spike and muscle potential (O'Gara et al., 1982). B) By seven days in the same animal, a MGF spike (dot) conducted up to, through, and out of the transplant region. Within the transplant, the spike was not accompanied by any detectable motor neuron or muscle potential. Note the relatively large amplitude and long duration of the spike in the transplant region, as well as the relatively long conduction time (approximately 3 ms) into and out of the transplanted VNC. C) By 27 days after transplantation in a Group I animal, the MGF spike (dot) appeared to be followed by motor activity in the transplant region. Abbreviations: A—anterior, P—posterior.
Figure 2. Non-invasive recordings of LGF responses to light tactile stimulation in three Group I animals. A) Two days after transplantation LGF spikes (evoked by touching segments just posterior to the transplant region) conducted up to, but not into, the transplant region (vertical bar). B) By four days after transplantation, one of three LGF spikes, evoked by light tactile stimulation to segments just anterior to the transplant region, was conducted into the transplant. This spike failed to conduct out of the transplant region. C) By five days after transplantation, LGF spikes were reliably conducted into and out of the transplant region. Note the relatively long conduction time through the transplant (2.8 ms). Abbreviations: A--anterior, P--posterior.
initial through-conduction the LGF spike amplitude and duration were near normal.

The typical pattern of MGF activity was not seen in the transplant region during the first week after re-establishment of MGF through-conduction (Fig. 1). That is, the MGF spike was not accompanied by any detectable motor neuron or muscle potential, and MGF spike amplitude and duration appeared slightly greater than normal.

Giant fiber conduction velocity within the transplant region for Groups I, II, IV, and V was always slower than in adjacent regions. Initially, in Group I mean conduction velocities for MGF and LGF within the transplant were 1.5 and 1.2 m/s, as compared to normal MGF and LGF velocities of approximately 6-8 and 4-6 m/s for posterior segments. After three weeks, MGF and LGF conduction velocities were 3.3 and 1.9 m/s, respectively (Fig. 3A). Initially, in Group II mean MGF and LGF velocities were 1.1 and 1.2 m/s, as compared to normal MGF and LGF velocities of approximately 7-11 and 4-5 m/s for anterior segments. By 30 days, MGF and LGF velocities had increased to 4.5 and 1.8 m/s, respectively (Fig. 3B). After 4-6 weeks velocities in the transplant remained approximately one-third to one-half normal values. Comparable values were obtained initially and at 28 days in Groups IV and V.

In all groups with transplanted VNC, there were initially long conduction times across each of the two junctions joining recipient and donor VNC. For example, in the MGF of one animal (Fig. 1B), the trans-junctional conduction times for a 2 mm distance were 3.7
Figure 3. Relationship between giant fiber conduction velocity in the transplanted VNC and time after initial through-conduction. A) Each point represents the mean conduction velocity (± SE) for the six animals in Group I. B) Each point represents the mean conduction velocity (± SE) of the eight animals in group II. The same worms were tested repeatedly throughout the six weeks after transplantation. Giant fiber conduction velocity in both groups increased but never attained normal values. Conduction velocities in normal animals were 7-11 m/s and 4-6 m/s for the MGF and LGF, respectively. MGF (solid circles), LGF (open circles).
DAYS AFTER THROUGH-CONDUCTION

A

CONDUCTION VELOCITY (m/s)

B

CONDUCTION VELOCITY (m/s)
Figure 4. Changes in giant fiber conduction time across the junctional region. Points indicate a mean ± SE (n = 28). Conduction times across the junction gradually decreased but did not attain values found in unoperated animals over the same distance. Conduction times over the same distance in normal animals were 0.3 and 0.4 ms for the MGF and LGF, respectively. MGF (solid circles), LGF (open circles).
TRANS-JUNCTIONAL CONDUCTION TIME (ms)

DAYS AFTER THROUGH-CONDUCTION
(anterior junction) and 3.8 ms (posterior junction), while corresponding values in the LGF (Fig. 2C) were 2.3 (anterior junction) and 2.5 ms (posterior junction). In adjacent unoperated VNC, MGF and LGF conduction times over the same distance remained essentially normal (0.3 ms and 0.4 ms, respectively). Because no statistical differences were seen between anterior and posterior trans-junctional conduction times in the same animal, or between animals of Groups I and II, all trans-junctional conduction times were combined (Fig. 4). As seen in Figure 4, trans-junctional conduction times gradually decreased and by six months after transplantation were approximately 0.8 ms for the MGF and 1.5 ms for the LGF. Trans-junctional conduction times from Groups IV and V were not included.

One unusual phenomenon observed in the junctional region was occasional "rebounding" of giant fiber spikes, similar to that reported in regenerating giant fibers of Lumbricus terrestris (Balter et al., 1980) and in whole body segment grafts of Eisenia fetida (Part I). Such rebounding involved giant fiber spike propagation up to the junctional region and rebound propagation along the same path. In some cases, there was an accompanying spike in the transplant region (Fig. 5A). Rebound spiking occurred in at least some animals in all groups and in all animals in Group V.

A second unusual phenomenon in the junctional region of Group IV and V animals was an apparent "cross-talk" between the giant fibers. In all cases this involved a LGF spike propagating up to the junction
Figure 5. Rebound spikes and apparent "cross-talk" in animals from Group V. A) A single anteriorly conducted LGF spike was initiated by tactile stimulation of the tail. After a 5.8 ms delay across the junction, a LGF spike was initiated in the transplant region (vertical bar). This spike appeared to then trigger a posteriorly conducted spike (dot) which, in turn, initiated another anteriorly conducted spike in the transplant and another rebound spike. B) Two anteriorly conducted LGF spikes (dots in lower record), initiated by tactile stimulation to the tail, were conducted up to the junction region. Each LGF spike appeared to trigger a posteriorly conducted MGF spike (dots in third trace from bottom).
and, after a 4-5 ms delay, initiation and propagation of a MGF spike in the opposite direction from the junction (Fig. 5B).

A third unusual phenomenon observed in only one animal (Group V) was an apparently spontaneous and rhythmic bursting of LGF spikes (Fig. 6). This bursting lasted approximately 45 min and was initiated at the junctional region as indicated by analysis of spike onset at higher sweep speeds.

Recovery of Sensory Function

In normal, adult Eisenia fetida with body lengths of 104 segments \( \pm 6 \) SD (\( n = 63 \)) MGF spikes were evoked by touch in segments 1-58 \( \pm 6 \) SD and LGF spikes by touch in segments 30 \( \pm 3 \) SD to the last segment. Thus, segments 30-58 represented the average overlap of MGF and LGF sensory fields.

In Group I, recovery of giant fiber sensitivity to touch in every segment of the transplant region involved only the LGF system. This recovery was first evident 5.2 days \( \pm 1.0 \) SD after transplantation. In all cases, the initiation of LGF spikes, as indicated by spike onset, occurred within the transplant region rather than in regions adjacent to the transplant (Fig. 7A). In some cases, this recovery of LGF touch sensitivity in the transplant region was established before LGF through-conduction. These results indicate that recovery of LGF sensitivity involved re-establishment of peripheral portions of sensory-to-giant pathways within the transplant rather than expansion of LGF sensory fields from adjacent segments into the transplant.
Figure 6. Rhythmic bursting of the LGF in a Group V animal five days after transplantation. Bursts of spontaneously generated LGF spikes (two to three spikes per burst) were detected in the transplant region (vertical bar) as well as at sites anterior (A) and posterior (P) to the transplant.
Figure 7. Examples of giant fiber responses to touch in the transplant region (vertical bar)  
A) In a Group I animal, a single LGF spike initiated by a touch stimulus (ST), propagated anteriorly and posteriorly out of the transplant region. B) In a Group II animal, a MGF spike (dot), evoked by touch, propagated anteriorly and posteriorly out of the transplant region.
Figure 8. Expansion of sensory field in the transplant region  A) In a Group I animal, LGF touch sensitivity was first evident (day 1) in the most posterior four segments of the transplant region; the anterior eight segments were insensitive. By day two, the LGF field was expanded to nine segments and by day three, to all segments of the transplant. Sensitivity was maintained for up to six months after transplantation.

B) In a Group II animal, MGF touch sensitivity was first evident (day 1) in the two most posterior segments; the anterior ten segments were insensitive. LGF spiking was also evoked in the most posterior segment. By day two, the MGF field expanded to all but the most anterior segment. By day three, and continuing up to six months, MGF sensitivity persisted in all segments.
Invariably, recovery of LGF touch sensitivity in the transplant region was not uniform; that is, only a few segments or portions of segments were touch sensitive. In some animals, these initially sensitive segments were at the end of the transplant (Fig. 8A), but in other animals segments in the middle of the transplant were initially sensitive. In all animals, the LGF sensory field expanded within one week to include all segments in the transplant region, although sensitivity was less than in normal posterior segments.

In Group II, recovery of giant fiber sensitivity to touch in every segment of the transplant region involved the MGF system (Fig. 7B). This recovery was first evident 9.2 days ± 2.8 SD after transplantation. The time to recovery in Group II was significantly greater (p < 0.001, df = 12, two-tailed Student t-test) than that in Group I.

Initially, as in Group I, giant fiber sensitivity in the transplant region was evident in only some segments or portions of segments. In some animals, these initially sensitive segments were at the ends of the transplant (Fig. 8B), but in other animals segments in the middle of the transplant were initially sensitive. Gradually, however, sensitivity developed in all segments of the transplant region. Although recovery of sensitivity involved the MGF in every segment, LGF and MGF spiking were often evoked by touching the first or last segment of the transplant region. Possibly the development of LGF sensitivity in these segments involved peripheral expansion of the LGF sensory field from adjacent normal segments. Although not quantified,
the sensitivity in every segment of the transplant region seemed less than sensitivity to touch in anterior segments of normal worms.

Restoration of giant fiber sensitivity in Group II animals was further investigated by obtaining intracellular recordings from the MGF. In normal posterior regions (segments 75-80) of Eisenia fetida small EPSPs (2-4 mV) were evoked in the MGF in response to electromechanically driven stimulation of the body wall. Such responses never initiated a MGF spike, even during repetitive stimulation. In anterior segments of normal worms, however, touching the body wall evoked larger EPSPs (up to 12 mV) in the MGF, and these were often sufficient to evoked a spike (Fig. 9A). In Group II animals, a single electromechanically driven stimulus to the body wall of the transplant region evoked 6-12 mV EPSPs in the MGF but these rarely triggered a spike. However, twin-pulse or repetitive stimulation usually evoked MGF spiking in the transplant region (Fig. 9B). If segments adjacent to the transplant region were touched, little or no depolarization occurred in the MGF of the transplant.

Sensory fields were also determined in Groups IV and V. In both groups sensitivity to touch was re-established in the transplant region 5-10 days after transplantation. In Group IV, MGF spiking was invariably evoked by touch to every segment of the transplant region. In Group V LGF spiking was evoked by touch to every segment of the transplant region, although occasionally MGF spiking was also evoked by touch to the first and last segments of the transplant region. Taken together these results indicated that the predominant pattern of
Figure 9. Intracellular recordings of MGF in responses to touching the body wall with an electromechanically-driven pin.  
A) In segment twelve of a normal animal, a single stimulus (dot) to a segment anterior to the recording site, evoked a large EPSP (up to 12 mV) and a single spike.  
B) In the transplant region (segments 75-87) of a Group II animal, two sequential stimuli (dots) to the body wall in the same segment as the recording electrode evoked an EPSP and MGF spike.
restored giant fiber sensory field in the transplant region invariably reflected that of the donor.

The possibility was then tested that the transplanted VNC from young animals may be more plastic in the pattern of functional restoration than adult VNC. This was done by transplanting anterior lengths of VNC (segments 10-34 or 10-40) from worms less than two weeks old (body weight = 50-100 mg) into posterior portions of adult recipient worms from which twelve segments of VNC had been removed (segments 75-87). A greater number of VNC segments was required from younger animals to span the denervated region in recipient animals. In the young donor animals, the MGF sensory field extended from segments 1-54 ± 4 SD and the LGF sensory field from segment 32 to the last segment ± 3 SD (n = 38). Thus, the transplant from these animals consisted of approximately 22 segments of VNC from within the MGF sensory field and the remaining 2-8 segments from an overlapping region of both MGF and LGF sensory fields.

In six of 31 recipients, transplanted VNC formed through-conducting and appropriate giant fiber connections with the adult recipient VNC. In 23 of the recipients, there were no signs of giant fiber through-conduction in the transplant region by four weeks after surgery. In the remaining two animals, segmental autotomy occurred near the transplant region.

Sensory function was tested in the six animals with giant fiber functional recovery. In all cases, the restored sensory field was essentially identical to that in the donor, MGF sensory field extending
throughout the transplant and the LGF field limited to three segments at one end of the transplant region.

Recovery of MGF-mediated Motor Responses

Motor neuron spikes were not detectable in non-invasive recordings from within the transplant region when MGF spikes were initially conducted through the transplant (Fig. 1B). Subsequent recovery of MGF-mediated motor activity was indicated by the reliable triggering of a presumed motor neuron spike at recording sites within the transplant. These spikes occurred approximately 1.0 ms after each MGF spike, as seen in normal MGF activity patterns. In Group I this recovery occurred 15.7 days ± 3.9 SD (n = 6), and in Group II 16.8 days ± 4.2 SD (n = 8), after transplantation. These values were not significantly different.

Initially, the motor neuron spikes in the transplant region were not accompanied by detectable muscle potentials, although MGF-mediated potentials were reliably detected at adjacent recording sites. After two to seven days, MGF-mediated potentials were first detected in the transplant region, although they lacked the smooth appearance of normal MGF-mediated muscle potentials. After 6-8 weeks, the potentials were smoother in appearance, and indistinguishable from those in normal worms. Essentially identical patterns of restoration of MGF-mediated motor activity were obtained from Groups IV and V.

The degree of facilitation of MGF-mediated muscle potentials in the transplant region of Group I and II animals was determined by
Figure 10. Non-invasive recordings of MGF spikes and accompanying muscle potentials in posterior segments with transplanted VNC. A) In a Group I animal, six weeks after transplantation, there was no facilitation of MGF-mediated muscle potentials in the transplant region (vertical bar). B) In a Group II animal, five weeks after transplantation, MGF-mediated muscle potentials showed marked facilitation in the transplant region, but no facilitation was evident in anterior (A) and posterior (P) recording sites adjacent to the transplant. In both records, paired MGF spikes (dots) were evoked by light tactile stimulation of the head.
electrically stimulating the MGF with twin-pulses (8 ms delay). In normal worms, the facilitation ratio of these potentials was $5.8 \pm 1.2$ SD (211 measurements from 63 animals) in segments 10-26 and $0.9 \pm 0.1$ SD (232 measurements from 63 animals) in segments 75-90. In Group I (Fig. 10A), 30-40 days after transplantation, the facilitation ratio in the transplanted region was $0.9 \pm 0.2$ SD (18 measurements from six animals), or essentially identical to that in normal posterior segments. In contrast, 30-40 days after transplantation in Group II (Fig. 10B) the ratio in the transplant region was $1.9 \pm 0.6$ SD (26 measurements from eight animals).

Facilitation of MGF-mediated muscle potentials was examined in simultaneous intracellular and surface recordings from four animals in Group II (Fig. 11A). In all cases, the degree of facilitation in intracellular records reflected that in extracellular records and the overall tendency for facilitation resembled that in anterior segments of normal animals (Fig. 11B).

In summary, there was a predictable sequence of restoration of giant fiber through-conduction, touch sensitivity, and MGF-mediated motor activity in the transplant region of Groups I and II animals (Figs. 12 and 13). The sequence and timing of functional restoration for the two groups were nearly identical, with the exception of much earlier restoration of sensory function in Group I. The basis for this difference was not addressed in the present study.
Figure 11. Simultaneous surface and intracellular recordings of MGF-mediated longitudinal muscle potentials  
A) In a Group II animal a pair of MGF spikes was evoked by just-threshold twin pulse electrical stimulation to segment 50. In the upper trace, the MGF spikes (dots) and accompanying muscle potentials were detected through the body wall in the transplant region (approximately segment 80). The lower trace shows facilitation of the corresponding muscle EPSPs recorded in the transplant region.  
B) In a normal animal a pair of MGF spikes (dots) and accompanying muscle potentials were detected in segment 20 (upper trace). The lower trace shows facilitation of the corresponding EPSPs in the same segment.
Figure 12. Time to functional recovery of escape reflex components in Group I animals. Restoration of sensory function in the transplant region occurred in less than one week and usually preceded establishment of LGF and MGF through-conduction. Restoration of MGF-mediated motor activity usually occurred 2-3 weeks after transplantation. In all cases, the functional integrity of these pathways was maintained for at least six months. The left end of each bar represents the mean recovery time (n = 6); the lines indicate the earliest recovery times.
FUNCTIONAL RECOVERY (Group I)

- LGF
- MGF
- SENSORY
- MOTOR

DAYS AFTER TRANSPLANTATION
Figure 13. Time to functional recovery of escape reflex components in Group II animals. Establishment of LGF and MGF through-conduction in the transplant region usually preceded sensory recovery. The time to sensory recovery was significantly slower than in Group I animals (Fig. 12). In all cases, the functional integrity of these pathways was maintained for at least six months. The left end of each horizontal bar represents the mean recovery time (n = 8); the lines indicate the earliest recovery times.
FUNCTIONAL RECOVERY (Group II)

DAYS AFTER TRANSPLANTATION

LGF

MGF

SENSORY

MOTOR
Behavior

Three stereotyped reflex responses were compared in Groups I and II, and normal worms. These were 1) rapid shortening responses to anterior or posterior touch, 2) setal responses to touch, and 3) forward and reverse peristaltic reflexes (Laverack, 1963; Mill, 1975; Gardner, 1976; Drewes, 1984).

In normal animals, an abrupt touch at either end of the animal evoked an end-to-end shortening. After transplantation in both groups, abrupt touch to either end of the animal evoked longitudinal shortening up to, but not beyond, the transplant region. Immediately after recovery of giant fiber through-conduction, as indicated by electrophysiological recordings, the animal shortened end-to-end except in the transplant region. Recovery of shortening within the transplant region occurred 3-4 weeks after recovery of giant fiber through-conduction, corresponding to the restoration of giant fiber-mediated muscle potentials in the transplant region.

Responses to anterior and posterior stimulation were accompanied by stereotyped setal movements. For example, in normal worms anterior tactile stimulation elicited setal retraction in anterior segments and setal protraction in posterior segments. By comparison in normal animals, posterior tactile stimulation elicited setal protraction in anterior segments and setal retraction in anterior segments. In Groups I and II, restoration of setal responses in the transplant region approximately coincided with restoration of
shortening responses. In Group I, the restored setal response to anterior stimulation was setal protraction and to posterior stimulation was setal retraction. In Group II, the restored setal response to anterior stimulation was setal retraction and to posterior stimulation was setal protraction.

Other reflex movements elicited by tactile stimulation in normal worms were forward peristalsis, initiated by gently stroking posterior segments, and reverse peristalsis, initiated by stroking anterior segments. In Group I, gentle stroking of the body wall in the transplant region invariably evoked forward peristalsis. In contrast, in Group II, stroking the body wall in the transplant region initiated reverse peristalsis. Forward and reverse peristaltic reflexes evoked by touching regions outside the transplant appeared normal.
DISCUSSION

Interconnections

Electrophysiological evidence in this study supports morphological evidence from the preceding study (Part II) showing that MGF and LGF in the transplanted VNC formed long-term and cell-specific connections with their respective counterparts in the recipient VNC. The specificity appeared independent of the anterior or posterior origin of the transplanted VNC, its orientation (up-side-down or anterior-posterior reversal), or age (young versus adult). Cell-specific regeneration of earthworm giant fibers has also been reported following single VNC transection in *Lumbricus terrestris* (Birse and Bittner, 1976; Balter et al., 1980; Birse and Bittner, 1981) and following VNC transection or grafting in *Eisenia fetida* (Part I). Thus, cell-cell specificity seems to be a general feature of earthworm giant fiber reconnections following a variety of experimental manipulations.

Behavioral observations suggest a similar specificity of reconnection in other central pathways, such as those mediating peristaltic reflexes. In normal earthworms, forward and reverse peristaltic locomotion can be reflexively initiated by gently stroking the posterior and anterior ends of the animal, respectively (Collier, 1939; Trueman, 1975; Gardner, 1976). In earthworms receiving anterior VNC transplants (Group I), stroking the transplant region invariably initiated reverse peristalsis originating in tail segments.
Conversely, in animals with posterior VNC transplants (Group II), stroking the transplant region initiated forward peristalsis originating in head segments. These observations suggest a specific reconnection of central pathways mediating forward and reverse peristaltic reflexes.

Electrophysiological evidence also indicated re-establishment of peripheral portions of giant fiber reflex pathways in animals receiving VNC transplants. In particular, touch sensitivity of the giant fibers as well as MGF-mediated motor neuron and muscle activity were re-established (Figs. 7 and 10). Presumably this re-establishment occurred following the en bloc transfer and subsequent regeneration by centrally located mechanosensory and motor neurons in the transplanted VNC. Irrespective of the anterior or posterior origin of the VNC transplant, properties of the re-established sensory and motor pathways always reflected those of the donor VNC. Specifically, segments receiving VNC of anterior origin exhibited MGF sensitivity to touch and facilitation of MGF-mediated muscle potentials, whereas segments receiving VNC of posterior origin exhibited LGF sensitivity to touch and no facilitation of MGF-mediated muscle potentials. The most likely explanation for these observations is a maintenance, after transplantation, of 1) central connectivity patterns involving sensory-to-giant fibers, 2) giant fiber-to-motor neuronal connectivity, and 3) facilitation properties of neuromuscular junctions associated with the MGF system.
Other motor components of escape responses (i.e., setal protraction or retraction) were established in the transplant region, as inferred from observations of these responses in the transplant region to tactile stimulation at the anterior or posterior end of the animal. In normal animals, when anterior regions of the worm were touched, setae protracted in posterior segments thus anchoring the animal’s posterior end during anterior withdrawal while setae in anterior segments retracted. When posterior segments of these worms were touched, setae in anterior segments protracted while setae in posterior segments retracted. In earthworms receiving transplanted VNC, restoration of setal responses in the transplant region occurred after approximately three weeks and in all cases the nature of such responses (i.e., protraction or retraction) reflected the origin of the transplanted VNC, as would be expected if central connections in setal reflex pathways were maintained after transplantation.

A comparable maintenance of central connections and appropriate peripheral regeneration can be inferred from behavioral studies in other systems. Detwiler (1923; 1949) studied restoration of swimming behavior following excision, anterior-posterior reversal, and re-implantation of spinal cord or hindbrain in larval Ambystoma. After several weeks, normal swimming behavior was restored suggesting reinnervation of swimming muscles. Presumably, this restoration also involved a maintenance of central connections required for the generation of motor outputs for swimming. Similarly, Straznicky (1963) and Narayanan and Hamburger (1971) made heteroptopic transplantations
(e.g., brachial to lumbosacral spinal cord) in embryonic chicks. In normal chicks the wings, which receive innervation from brachial spinal cord, move synchronously while the legs, which receive innervation from lumbrosacral spinal cord, move alternately. Animals receiving heterotopically transplanted spinal cord showed synchronous leg movements instead of alternating leg movements, thus indicating a maintenance rather than rearrangement of central-to-motor connections after transplantation.

The mechanisms for the formation of peripheral reconnections in transplanted earthworm VNC were not examined in the present study, but two likely possibilities were the formation of new distal fibers by outgrowths from damaged sensory and motor neurons in the transplanted VNC or fusion between proximal and homologous distal portions of severed axons. The latter seems less likely, however, since many lateral nerves appeared to degenerate after transplanted VNC to body wall were often irregularly arranged and ventral in position (cf. Fig. 6, Part II).

This unusual orientation of peripheral connections occurred whether the transplanted VNCs were matched with peripheral target tissue from the same or different body regions. Since formation of these connections was always accompanied by the functional restoration in sensory and motor pathways, it may be inferred that peripheral target tissues were successfully reinnervated and that specificity of target selection by transplanted sensory and motor neurons was broad enough to include serially or laterally homologous tissues from
different body regions. Similarly, previous studies have indicated a specificity of reconnections between regenerating motor neurons and serially homologous, as well as laterally homologous, target muscles in arthropods (Young, 1973; Bate, 1976; Fourtner et al., 1978; Ely and Velez, 1982; Hunt and Velez, 1982).

In earthworms, once peripheral connections were established between transplanted motor neurons and body wall muscle, functional properties of neuromuscular transmission (i.e., facilitation of MGF-mediated muscle potentials) appeared unchanged in comparison to those of the original transplant segments. For example, 9-10 months after transplantation of anterior VNC into posterior segments facilitation of MGF-mediated longitudinal muscle potentials was a persistent characteristic in the transplant region (Fig. 10). Thus, there appeared to be little, if any, long-term alteration of motor neuronal properties following reinnervation of longitudinal muscle.

Changing Properties of Transplanted Giant Fibers

At about the time that through-conduction of giant fiber spikes was established between transplanted and recipient VNC, giant fiber diameters in the junctional region were normal to twice normal diameter. Nevertheless, conduction velocities were much slower than normal (less than 1.0 m/s as compared to 8.0 m/s and 6.0 m/s for normal MGFs and LGFs, respectively). Subsequently, giant fiber conduction velocities across the junction increased, in some cases threefold (Fig. 3), but there were no accompanying changes in giant fiber diameter in
this region. Such increases in velocity could indicate any of a number of alterations in giant fiber membrane or electrical properties, such as membrane excitability or cable properties. Changes in conduction properties of regenerating neurons have been reported for outgrowing tips of cockroach giant axons, in which an initially Ca\(^{++}\)-dependent spike was gradually replaced by a Na\(^{+}\)-dependent spike (Mieri et al., 1981).

Within the transplant region, giant fiber morphology was initially normal but after 4-5 weeks was characterized by the presence of numerous branch-like extensions (Part II). Despite these modifications, giant fiber conduction velocities within the transplant remained relatively constant (Fig. 3) and afferent and efferent characteristics of giant fiber reflex pathways were maintained. Thus, transplanted giant fibers exhibited considerable morphological plasticity without obvious physiological consequences.

For the first few days after establishment of giant fiber through-conduction, some unusual electrophysiological phenomena were evident in the junctional region between transplanted and recipient VNC. One of these was a rebounding of giant fiber spikes (Fig. 5A), similar to that reported during early stages of regeneration in severed giant fibers of *Lumbricus terrestris* (Balter et al., 1980) and in grafted VNCs in *Eisenia fetida* (Part I). Balter et al. (1980) suggested that such rebounding could occur if a giant fiber spike produced an excitation in the regenerating area which was sufficiently long to outlast the refractory period in adjacent membranes, thereby
re-exciting these fibers. Another unusual phenomenon was the apparent "cross-talk" between the LGF and MGF (Fig. 5B). Possibly, this "cross-talk" resulted from some type of direct electrical interaction between the LGF and MGF in the junction (e.g., field effects or an electrical synapse). Similar "cross-talk", as well as rebounding, have been reported in association with chemically-induced or disease-related demyelination in mammalian nerve fibers (Rasminsky, 1980). It is conceivable that a loss or reduction of the myelin-like sheath surrounding earthworm giant fibers (Taylor, 1940; DeRobertis and Bennett, 1956; Hama, 1959; Gunther, 1976) may predispose the giant fibers toward such electrical interactions. An alternative explanation, however, for "cross-talk" in earthworm giant fibers was mediation by some indirect excitatory pathway involving an interposed neuron. For example, one identifiable interneuron has been shown to be mutually associated with MGF and LGF collaterals in some segments in Lumbricus terrestris (Gunther and Walther, 1971; Gunther and Schurmann, 1973). There is, however, no physiological data supporting any LGF to MGF interaction in normal animals.

In summary, physiological, anatomical, and behavioral results from VNC transplantation experiments indicated that sensory, interneuronal, and motor components of earthworm escape reflex pathways in the transplant formed appropriate central and peripheral reconnections to target cells. The specificity of these reconnections was sufficiently broad to include serially and laterally homologous target cells or tissues from other body regions. Since numerous characteristics of
escape reflex function appeared unchanged following transplantation, we conclude that escape reflex function can not be altered by this procedure or, alternatively, that differentiated peripheral tissues failed to induce such changes.
SUMMARY

Twelve segments of ventral nerve cord (VNC) from donor earthworms, *Eisenia foetida*, were transplanted into recipient worms from which a comparable length of VNC had been removed. Donor VNC was taken either from segments 10-22 (i.e., within the MGF sensory field) or from segments 75-87 (i.e., within the LGF sensory field). Denervated regions in recipient animals were either segments 10-22 or 75-87. Irrespective of the origin of the transplanted VNC or site of transplantation, specific MGF-to-MGF and LGF-to-LGF connections were established between transplanted and recipient VNC, as indicated by through-conduction of MGF and LGF spikes within 4-10 days after transplantation. For the first few days after through-conduction, properties of giant fiber conduction included: rebounding of giant fiber spikes, delays in conduction across the junction between transplanted and recipient VNC, and slow conduction velocities in the transplant (one-fifth to one-fourth normal). Thereafter, the conduction of giant fiber spikes was reliable, with gradual decreases in trans-junctional conduction times and increases in conduction velocities in the transplant. However, the velocities never reached normal values.

Restoration of the functional integrity of afferent-to-giant fiber pathways was indicated by initiation of giant fiber spiking in the transplant region in response to light tactile stimulation to the body wall. By 8-14 days after transplantation, touching segments 75-87 in
animals receiving anterior VNC transplants evoked MGF spikes; whereas 4-6 days after transplantation, touching segments 75-87 in animals receiving posterior VNC transplants evoked LGF spiking. Restoration of MGF-mediated motor pathways in the transplant occurred in all groups as indicated by the reappearance of MGF-mediated motor activity by 17-22 days after transplantation. In all cases, the restored functional properties of the afferent and motor pathways in the transplant region reflected the origin of the transplanted VNC.
REFERENCES


PART IV. EFFECTS OF FOOD DEPRIVATION ON THE FUNCTIONAL DEVELOPMENT OF GIANT NERVE FIBERS IN THE EARTHWORM, *Eisenia foetida*
INTRODUCTION

Ontogenetic growth of animals encompasses every organ system, including the nervous system. The achievement of normal nervous system growth, as well as somatic growth in general, depends upon a sufficient level of food availability. In mammals, insufficient levels of food, especially during so-called "vulnerable periods" of nervous system development, may cause permanent stunting effects, including possible decreases in nerve cell number or size (Dodge et al., 1975; Dobbing, 1976; Winick, 1976).

Although there is an extensive information base regarding food-deprivation effects on the development of the whole brain or specific neuronal populations, there has been little study of the effects of food deprivation on the functional development of the giant nerve fiber systems, which are found in many invertebrates and lower vertebrates. Considering the utility of giant fiber systems for studies of developmental neurobiology (Kimmel and Model, 1978; Kimmel and Eaton, 1976; Govind and Lang, 1976; Lang et al., 1977), it seems appropriate to examine possible food-deprivation effects on the growth and functional development of a selected giant fiber system.

The giant nerve fibers of the lumbricid earthworm, Eisenia foetida, offer several experimental advantages for such studies. First, these earthworms are easily reared in the laboratory under a variety of growth conditions and reach the adult stage in a few weeks (Kaplan et al., 1980; Neuhauser et al., 1980a). Second, by means of
non-invasive electrophysiological recordings, long-term and repeated assessments of the functional development of the giant fibers can be obtained throughout postembryonic growth (O'Gara et al., 1982). The objectives of this study were as follows: 1) to examine possible deleterious effects of various levels of food deprivation on postembryonic development of earthworm giant nerve fibers, and 2) to determine the relationship between patterns of giant fiber growth and somatic growth under various conditions of food deprivation.
MATERIALS AND METHODS

Animals and Maintenance

Forty-two newly hatched earthworms (mean weight = 5.5 mg ± 2.7 SD), *Eisenia foetida* Savigny, were collected from cocoons of laboratory-bred worms and divided into seven groups. The worms in each group were individually reared in separate containers (15 X 100 mm glass petri dishes). As shown in Table 1, the rearing media for these groups contained various quantities of food (i.e., horse manure); the microbial content of the manure is the worms' food source (Neuhauser et al., 1980b). The manure, after being frozen to decrease insect populations, was thawed, air-dried, and grated through a screen (mesh size 1/6 X 1/16 in.) to reduce particle size. Soil was included in the medium of each group (except Group VII) because soil factors may help promote the conversion of manure to earthworm biomass (Kaplan et al., 1980; Neuhauser et al., 1980b). The amounts of distilled water added to each medium were sufficient to hydrate the manure to a water content (w/w) of 75% and the soil to 27%. The media contents for each group were thoroughly mixed together as a batch, incubated overnight at 25°C, and divided equally among the six individual animal dishes. The resulting amount of manure, soil, and water per worm (i.e., per dish) is shown in Table 1. The original media for Groups I-VI were not renewed throughout the experiment (56 days).

In Group VII, each of the six animal dishes contained 1 ml of distilled water and a small wedge of Sylgard (Dow Corning) which
Table 1. Composition of media with various levels of food deprivation

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<th>I</th>
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<th>IV</th>
<th>V</th>
<th>VI</th>
<th>VII</th>
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<tbody>
<tr>
<td>Number of animals/group</td>
<td>5^a</td>
<td>6</td>
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<td>5^a</td>
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<tr>
<td>Horse manure (g dry matter/worm)</td>
<td>5.0</td>
<td>2.5</td>
<td>1.25</td>
<td>0.5</td>
<td>0.5</td>
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<tr>
<td>Soil (g dry matter/worm)</td>
<td>30.0</td>
<td>30.0</td>
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<tr>
<td>Distilled water (ml/worm)</td>
<td>26.0</td>
<td>18.5</td>
<td>14.75</td>
<td>12.5</td>
<td>11.3</td>
<td>11.0</td>
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^aOne animal died of causes unrelated to the experimental treatment.
provided an irregular surface for crawling or contact. The dishes were sealed with parafilm to prevent escape. Each dish was cleaned, dried, and remoistened with distilled water twice a week throughout the experiment.

**Testing Procedure**

Newly hatched worms were weighed and the medial giant fiber (MGF) and lateral giant fiber (LGF) conduction velocities were measured using a non-invasive electrophysiological testing procedure (O'Gara et al., 1982). Measurements of body weight and conduction velocity were made every seven days for four weeks, and every two weeks thereafter. A mean of three to six conduction velocity measurements (for MGF and LGF) was obtained from each worm on each of the test days. All velocity measurements were made between segments 25 and 75 at a temperature of 21-23°C. After the 10-15 minute period required to obtain these measurements, each worm was returned to its individual dish and maintained in the dark at 25°C. After final testing, statistical differences between groups were determined using a one-way analysis of variance with harmonic means (Keppel, 1973). Subsequent tests (least significant difference test = LSD test) were then used to determine significant group differences with respect to giant fiber conduction velocity.

To test whether the effects of food deprivation on development were reversible, some of the worms from Groups IV and VI were transferred after nine weeks into a food-abundant recovery medium. The
other worms (controls) in these two groups remained in their original media. Conduction velocity and body weights were then measured weekly.

In several groups diameters of living giant fibers were measured after final testing. Procedures for obtaining these measurements have been described previously (O'Gara et al., 1982).
RESULTS

Somatic Growth in Relation to Food Deprivation

As shown in Figure 1, considerable differences in somatic growth curves were seen in relation to various levels of food deprivation. Worms in Group I showed the greatest overall increase in somatic growth. The maximal rate of body weight gain in this group (approximately 160 mg/wk) occurred between days 14 and 28. These worms reached sexual maturity (i.e., clitellum formation) between days 21 and 28 and attained a mean body weight of 786 mg by day 56. More gradual increases in body weight continued after day 56. These patterns of somatic growth were comparable to those obtained in previous studies of normal earthworm development (Neuhauser et al., 1980b; O'Gara et al., 1982).

Groups II-V showed progressively lower levels of somatic growth, corresponding to increased levels of food deprivation. Worms in Groups II and III eventually reached sexually maturity between days 35 and 42, but none of the worms in Groups IV and V reached sexual maturity by day 56. Worms in Group VI, which were reared in soil only, showed merely slight increases in body weight (mean = 19 mg by day 56).

Worms in Group VII (no manure or soil) showed steady losses in body weight with time. By day 21, the mean body weight was 2 mg, or approximately 40% of the mean weight at hatching. Four of the worms in this group died between days 28 and 25; the remaining two worms died between days 36 and 42.
Figure 1. Long-term effects of various levels of food deprivation on body growth. Growth data are shown only for Groups I-VI, with numbers in parentheses indicating the grams of dry material contained in each worm's dish (see Table 1). With each decrease in the level of food, from 5.0 g manure (Group I) to 0.1 g manure (Group V), there was a marked depression in the corresponding growth curve. Worms in Group VI (open triangles) reared only in soil, showed the smallest increases in body weight. Worms in Group VII, reared in no soil or manure, gradually lost weight after hatching and were not graphed (see text for further explanation). At hatching (day 0) the mean body weight for each group ranged from 4-7 mg.
800i
600-
400-
200-
0-
MEAN BODY WEIGHT (mg)

DAYS AFTER HATCHING

I (5.0g)
II (2.5g)
III (1.25g)
IV (0.5g)
V (0.1g)
VI (0.0g)
Functional Development of Giant Fibers in Relation to Food Deprivation

As seen in Figures 2 and 3, age-related increases in giant fiber conduction velocity were seen in each of the seven groups. In Group I, the most rapid increase in giant fiber conduction velocity was seen during the first week after hatching, with MGF increasing in velocity from 2.0 m/s to 4.0 m/s and LGF from 1.2 m/s to 2.3 m/s. After the initially rapid increase in MGF conduction velocity, a much slower rate of increase was observed between days seven and 21, thus resulting in the slight inflection shown in Figure 2A. A very slight inflection in the increase in LGF conduction velocity was evident. Both MGF and LGF conduction velocities continued to increase at slower rates from day 28 to day 56. By day 56 MGF conducted at 2.8 m/s and the LGF at 4.7 m/s. These patterns of giant fiber development were comparable to those obtained in normal developing earthworms (O'Gara et al., 1982).

The patterns of age-related increases in giant fiber conduction velocity in Groups II-IV were nearly identical to those of the worms in Group I, despite their food deprivation and low levels of somatic growth (Figs. 2B and 3B). All intermediate groups showed initially rapid increases in giant fiber conduction velocities through day seven followed by slower rates of conduction velocity increase through day 56.

To statistically compare conduction velocity differences in the various groups we selected data from one day (day 42 after hatching) for a one-way analysis of variance. The analysis indicated significant differences in MGF velocity (p < 0.001, F = 15.98, df = 30) and LGF
Figure 2. Effects of various levels of food deprivation on age-dependent changes in MGF conduction velocity. (A) Well-fed (Group I) worms were compared with extremely food-deprived worms (Groups VI and VII). All three groups showed similar increases in MGF conduction velocity during the first few days after hatching, but beyond one week MGF velocities in Groups VI and VII were much lower than in Group I. (B) Worms in Groups II-V (intermediate food levels of food deprivation) were compared to one another and to Group I worms. Increases in conduction velocity in Groups II, III, and IV were similar to one another and to Group I. Velocity values in Group V were consistently lower than in other intermediate groups. Standard error mean values are not shown, but were within the range of 0.09-0.34 m/s for all groups. Graph symbols are the same as in Figure 1 with the addition of o--o for Group VII.
Figure 3. Effects of various levels of food deprivation on age-dependent changes in LGF conduction velocity. (A) Well fed worms (Group I) and extremely food-deprived worms (Groups VI and VII) were compared. Only slight increases in LGF velocity were seen in extremely food-deprived worms, but nearly a fivefold increase in velocity was seen in well fed worms. (B) Worms in Groups II-V (intermediate levels of food deprivation) were compared with one another and with Group I worms. Increases in conduction velocity in Groups II, III, and IV were similar to one another and to Group I, but velocity values in Group V tended to be lower than in other intermediate groups. Standard error mean values are not shown, but were within the range of 0.02 -0.20 m/s for all groups. Graph symbols are the same as in Figures 1 and 2.
velocity \((p < 0.001, F = 32.67, df = 30)\). Subsequent tests (LSD test) revealed the following differences between specific groups with respect to MGF, as well as LGF, conduction velocity: Groups I, II, III, and IV differ significantly \((p = 0.05)\) from Group VI.

To determine if the age-related conduction velocity increase in food-deprived animals were due to growth in fiber diameter (as in the case in normally developing well fed animals), the giant fiber diameters of three animals were measured in each of Groups III and V. For Group III, mean conduction velocities and corresponding diameters on day 63 were MGF = 7.5 m/s and 21.2 \(\mu\)m; LGF = 5.0 m/s and 12.0 \(\mu\)m. For Group V, mean conduction velocities and corresponding diameters on day 71 were MGF = 6.0 m/s and 15.6 \(\mu\)m; LGF = 3.2 m/s and 8.7 \(\mu\)m. These values fall within the calculated 95% confidence limits of previously established conduction velocity-versus-diameter plots for MGF and LGF (cf. Fig. 9, O'Gara et al., 1982). Therefore, we conclude that although severe levels of food deprivation stunted rates of giant fiber conduction, there was no apparent alteration in the predicted velocity-versus-diameter relationship in these animals.

Worms reared in soil only (Group VI) showed initially rapid increases in MGF conduction velocity. At seven days after hatching, the MGF conducted at 3.8 m/s, or nearly identical to the velocity of worms in Group I (Fig. 2A). Between days seven and 21, the MGF velocity appeared to decrease slightly but then gradually increased. By day 56, the MGF conduction velocity had reached 5.0 m/s, or slightly more than twice the velocity at hatching but much slower than the worms in Group
1. Increase in LGF conduction velocity followed a somewhat similar pattern, reaching a value of 2.4 m/s by day 56.

Giant fiber-mediated escape withdrawal in Groups I-VI appeared qualitatively normal in giant fiber conduction velocity. The peak MGF conduction velocity was 2.9 m/s at day three and the peak LGF conduction velocity was 1.7 m/s at day seven. Both MGF and LGF velocities then steadily decreased. By day 28, MGF and LGF velocities were 2.3 m/s and 1.3 m/s or approximately equal to velocities of newly hatched worms. The gradual decrease in giant fiber conduction velocity seen between days seven and 28 was accompanied by an increased tendency for apparent motor deficits in the MGF and LGF reflex systems. Often, MGF spikes were not accompanied by their characteristic motor neuron spike and muscle potential (Fig. 4). Also, high-frequency LGF spikes were often not accompanied by muscle potentials. Coincident with this apparent failure to evoke muscle activity was a failure to evoke rapid escape shortening, even in response to high-frequency trains of giant fiber spikes. In no animal did we observe failure to initiate giant fiber spikes. In addition, capabilities for peristaltic locomotion appeared normal at least through day 28.

Comparison of the Developmental Effects of Food Deprivation:

"Dose-response" Relationships

The pattern of functional development of giant fibers remained similar within a wide range of food-deprivation levels (Figs. 2 and 3) despite marked differences in somatic growth for each group (Fig. 1).
Figure 4. Medial giant nerve fiber (A) and LGF (B) activity 28 days after hatching in a starved worm (Group VII) from four simultaneous recording sites near the middle of the animal. (A) Very light tactile stimulation to the head evoked three monophasic MGF spikes which were not accompanied by a motor neuron spike or muscle potential which always accompany each MGF spike in normal animals. For comparison, (C) shows a normal MGF spike (dots) followed by a motor neuron spike and muscle potential from a newly hatched worm. (B) Light tactile stimulation of the tail of the starved worm evoked a high-frequency train of all-or-none LGF spikes. The diphasic spikes were conducted anteriorly at a velocity of approximately 1 m/s. Despite the high frequency of firing, the spikes were not accompanied by large muscle potentials which typically follow similar trains of LGF spikes in normal animals. The relatively long duration and slow conduction velocity of MGF and LGF spikes in these starved worms were quite comparable to those of late embryonic or newly hatched worms (cf. Figures 7 and 10, O'Gara et al., 1982).
Figure 5 shows the "dose-response" relationship for somatic growth (i.e., body weight) and giant fiber conduction velocity at day 42. A marked stunting in body weight was seen as the food level decreased from 2.5-0.5 g manure per worm. In contrast, for both MGF and LGF systems, no apparent differences in conduction velocities were seen between 5.0 and 0.5 g manure per worm. We conclude, therefore, that giant fiber growth (as indicated by increased conduction velocity) is much less vulnerable to food deprivation than is somatic growth.

Reversibility of Food-deprivation Effects on Giant Fiber Development

To test for the reversibility of extreme food-deprivation effect on giant fiber development, three worms from Group VI (reared in soil only) were transferred on day 63 after hatching to an abundant food supply (i.e., 20 g moist manure per worm). The other two worms (controls) in this group remained in their original medium. Before reversal, giant fiber conduction velocities (MGF = 5.0 m/s; LGF = 2.5 m/s) and body weight (17.8 mg) in the recovery group were essentially identical to those of the control group (Fig. 6).

After four weeks in an abundant food supply, mean body weight increased to 648 mg (range: 568-795 mg), as compared to only 11.3 mg (range: 7.4-15.2 mg) in the control worms. Also, after four weeks the mean MGF and LGF conduction velocities in recovery worms increased to 7.9 m/s (range: 7.5-8.3 m/s) and 4.3 m/s (range: 4.0-4.7 m/s), respectively. These values are well above the mean conduction velocities of the control group (MGF = 4.7 m/s, range: 4.5-4.9 m/s; LGF
Figure 5. Relationship between giant fiber conduction velocity and level of food deprivation in 42-day-old animals. Medial giant fiber (solid circles) and LGF (open circles) conduction velocities were essentially unaffected by food levels ranging from 5.0-0.5 g manure/worm, but marked decreases in velocities occurred at levels below 0.5 g. The SEM values for mean conduction velocities are not indicated on the graph, but for all concentrations SEM values were within the range of 0.2-0.3 m/s for MGF and 0.1-0.2 m/s for LGF. Body weight (solid triangles) was markedly less at each food level below 5.0 g. Standard deviations for the six mean body weight values, from highest to lowest concentration, were 79, 128, 96, 27, 18, and 9 mg, respectively.
Figure 6. Recovery from the effects of extreme and prolonged food deprivation. The five worms used in this experiment were previously maintained for 63 days after hatching in a medium of soil only (Table 1). Three of these food-deprived worms were selected as a recovery group (solid lines) and transferred (day 0 on the graph) to a medium containing abundant food (20 g moist manure). The other two food-deprived worms comprised a control group (dotted line) and remained in soil. (A) Body weights increased greatly in the recovery group, but not in the control group. (B) Medial giant nerve fibers (solid circles) and LGF (open circles) conduction velocities increased in the recovery group but not in the control group.
= 2.5 m/s, range: 2.2-2.7 m/s). Corresponding measurements of giant fiber diameter in the three recovery worms indicated that mean diameters had increased to 21.4 μm (MGF) and 13.9 μm (LGF), as compared to 11.9 μm (MGF) and 5.0 μm (LGF) for the two control worms. All these mean fall within the 95% confidence intervals of the predicted velocity-versus-diameter relationship (O'Gara et al., 1982). These results indicate that the stunting of giant fiber development in food-deprived animals, as well as the stunting of somatic growth, was rapidly reversible.

The results in Figure 6 raise the question of whether the increases in giant fiber conduction velocity and somatic growth during recovery from food deprivation are necessarily obligatory or coupled. To test this possibility, three worms from Group IV were transferred on day 63 after hatching to an abundant food supply (i.e., 20 g moist manure per worm). The other three worms (controls) in this group remained in their original medium. As shown in Figure 7, MGF and LGF conduction velocities in the reversal group remained nearly identical to those in the control group (MGF = 7.8 m/s, range: 7.6-8.3 m/s; LGF = 5.0 m/s, range: 4.6-5.3 m/s). In contrast, body weight of the recovery worms increased from 130 mg (range: 87-143 mg) before reversal to 656 mg (range: 500-844 mg) three weeks after reversal. These results indicate that marked increases in somatic growth can occur without obligatory increases in giant fiber conduction velocity.
Figure 7. Recovery from the effects of moderate but prolonged food deprivation. All six worms used in this experiment were previously maintained for 63 days after hatching in a medium containing a small amount of manure (0.5 g; Table 1). Three of these moderately food-deprived worms were selected as a recovery group (solid line) and transferred (day 0 on the graph) to a medium containing abundant food (20 g moist manure). The other three worms comprised a control group (dotted line) and remained in their original medium. (A) Body weight increased greatly in the recovery group, but not in the control group. (B) Medial giant nerve fiber (solid circles) and LGF (open circles) conduction velocities in the recovery group remained essentially identical to those in the control group.
MEAN CONDUCTION VELOCITY (m/s)  MEAN BODY WEIGHT (mg)

A

B

DAYS BEFORE
DAYS AFTER RECOVERY

14 14
7 7
0 0
21 21

2 2
4 4
6 6
8 8

200 400 600

LGF  MGF

173
DISCUSSION

Relationship of Giant Fiber Growth to Somatic Growth

In well-fed earthworms conduction velocity of the giant nerve fibers was age-dependent, increasing approximately fivefold from hatching to maturity. In both the MGF and LGF the patterns of postembryonic changes in conduction velocity were characterized by a rapid increase in velocity during the initial week after hatching, and then by a more gradual increase continuing for several weeks thereafter. When weekly rates of increased velocity (i.e., slopes in Figs. 2A and 3A) were estimated and then plotted as shown in Figure 8, two distinct peaks of velocity increase were evident. During the first peak, from days 0-7, the velocity increase was greater than at any other time after hatching. This rapid increase in velocity occurred well before the peak in somatic growth (Fig. 8A). Electrophysiological data from various embryonic stages (O'Gara et al., 1982) suggest that this first peak of rapidly increasing conduction velocity begins during late embryonic development, that is, when the giant nerve fibers are completing their formation along the length of the ventral nerve cord (Prosser, 1933).

Comparison of well-fed with extremely food-deprived worms (Fig. 8B) indicated little effect of food deprivation after hatching on the initial peak of rapidly increasing MGF conduction velocity (a slight depression of the LGF conduction velocity increase may have occurred). A likely explanation for continued giant fiber growth depends on the utilization of food reserves accumulated while in the cocoon. Prosser
Figure 8. Age-dependence of giant fiber growth rate and somatic growth rate in well-fed and extremely food-deprived worms. Giant fiber growth rates were determined by estimating the weekly slopes of MGF and LGF conduction velocity increases from Figures 2(A) and 3(A). Somatic growth rates were determined by estimating weekly slopes of weight gains from Figure 1. (A) In Group I (well-fed) worms two peaks in MGF growth rate (solid circles and solid lines) and LGF growth rates (open circles and solid lines) are evident with the second peaks coincident with the peak in somatic growth (dashed line). (B) In Group VI (soil only) worms the two peaks in giant fiber growth rate are still evident, although the second peaks are lower than in (A). No peaks in somatic growth are seen.
(1933) observed intake of albumen in early embryos during spontaneous contraction and ciliary movement of the stomodaeal region. In addition, we have observed a significant quantity of albumen in the digestive tract of newly hatched worms. Whatever the explanation for continued giant fiber growth, it is clear that even without any exogenous food available to newly hatched worms, conduction velocity increased during the first few days after hatching without concomittant increases in body weight (Figs. 1, 2A, and 3A). Further evidence for this apparent independence of giant fiber growth from somatic growth was obtained when worms with stunted body weights, but normal giant fibers (Group IV), were placed into abundant food. The results showed significant somatic growth, but no changes in giant fiber conduction velocity (Fig. 7). Taken together, these results demonstrate the relative priority of giant fiber growth in relation to somatic growth when good levels are low, and the independent control of giant fiber growth from somatic growth. Without further study, it remains uncertain whether this priority of growth involves only the giant fibers or whether it is a general feature of central nervous system growth.

As seen in Figure 8A, a second and broader peak in giant fiber growth rate was evident 2-5 weeks after hatching in well-fed worms. This second peak, although coincident with the obvious spurt in somatic growth in well fed worms, occurred without a somatic growth spurt in extremely food-deprived animals (Fig. 8B). These results are also
consistent with the idea of independent control and relative priority of giant fiber growth during prolonged periods of food deprivation. However, Figure 6 demonstrates that when food became abundant, even at a considerably late time, both giant fiber growth and somatic growth occurred at rates similar to those seen in well-fed worms 2-5 weeks after hatching. These results indicate the failure of long-term food deprivation to permanently stunt giant fiber growth and indicate the general plasticity of somatic and giant fiber growth in earthworms.

Our demonstration of independent control of giant fiber growth and somatic growth in earthworms may have general relevance to a number of other studies on the development of giant fiber systems. For example, Schwartz (1974) correlated age-dependent increases in the diameter of the Mauthner cells in hatchery-reared trout with increases in body length, noting a tenfold increase in Mauthner cell diameter and a 20-fold increase in body length during a three year period. Govind and Lang (1976) found a similar correlation of giant axon growth (increasing diameter) with somatic growth in laboratory-reared lobsters with a fourfold increase in diameter and a 20-fold increase in rostrum-to-telson length occurring from early juveniles to adult stages. By comparison we have found approximately a fivefold increase in earthworm giant fiber diameter (and velocity) and greater than 100-fold increase in body weight in well fed, laboratory-reared earthworms. However, during food deprivation age-related increases in giant fiber growth persisted without concomitant somatic growth. Thus,
within a wide range of food availability in laboratory-reared earthworms, giant fiber conduction velocity remained a much more reliable indicator, or predictor, of age than did somatic growth. Consequently, determination of giant fiber conduction velocity may prove a useful tool for estimating the age in field-collected worms.

Effects of Starvation on Giant Fiber Function

During extreme food deprivation (e.g., worms reared only in soil) giant fiber conduction velocities remained consistently lower than in well fed worms (Figs. 2A and 3A). Nevertheless, the values for velocity and diameter in these animals appear to fit the predicted velocity-versus-diameter relationship for normally developing animals (O'Gara et al., 1982). Therefore, the effect of extreme food deprivation may simply be a stunting of giant fiber diameter. However, because of the inaccuracies of fiber diameter measurement at the light microscopic level, it is possible that more subtle effects of food deprivation may also accompany the stunting of fiber diameter. For example, there could be anomalies in the growth and development of the sheath that surrounds each giant fiber (Hama, 1959; Gunther, 1976) or slight alterations in the electrical resistance of the segmentally arranged septa in the giant fibers (Kao and Grundfest, 1957; Kensler et al., 1979; Brink and Barr, 1977).

In completely starved worms, after an initial increase in giant fiber velocity, the velocity gradually decreased over 3-4 weeks to values typical of newly hatched worms (Figs. 2A and 3A). Despite the
decrease in velocity and continual loss of body weight, the capability for giant fiber mediated escape in response to light tactile stimulation persisted for several weeks. Although such responses appeared to be qualitatively similar to those of well fed worms, it is possible that subtle changes in the quantitative aspects of motor response occurred, as has been shown during starvation in other invertebrates (Hoyle, 1954). In earthworms, the only apparent change in escape responses occurred after several weeks of complete starvation and this involved an intermittent failure to evoke rapid escape responses. In the case of the MGF system, this failure did not appear to involve the afferent side of the escape reflex because we were always able to evoke high-frequency trains of MGF spikes in response to tactile stimulation of the head. Rather, there appeared to be failure to initiate the motorneuron spike and muscle potential that normally follow each MGF spike. Similarly, in the case of the LGF, high-frequency spikes always were evoked in response to tactile stimulation of the tail, indicating the afferent side of the reflex was functional. However, no muscle potentials that normally follow high-frequency trains of LGF spikes were detected, again suggesting failure somewhere along the efferent side of the reflex. Although failure of the escape reflex was symptomatic for a few days prior to the death of starving worms, throughout most of the starvation period giant fiber spiking consistently evoked motor response. This maintenance of functionally intact escape reflexes during food
deprivation might be expected, given the wide range of food availability in the natural environment and the importance of the rapid escape reflex to the animal's survival.
SUMMARY

Non-invasive electrophysiological recording methods were used to study the effects of prolonged food deprivation on the postembryonic patterns of giant fiber growth, as indicated by age-dependent changes in giant fiber conduction velocity and diameter, in the earthworm, Eisenia fetida. In addition, giant fiber growth was compared to patterns of somatic growth, as indicated by increases in body weight. Within a wide range of food deprivation levels, normal age-dependent increases in conduction velocity and diameter occurred in spite of marked stunting of somatic growth. Stunting of giant fiber velocity and diameter occurred only during severe food deprivation, but giant fiber spikes and associated rapid escape responses were still readily evoked. The stunting effects of prolonged and severe food deprivation upon giant fiber conduction velocity and diameter were readily reversed by replenishing food. The results demonstrate the persistence of rapid escape reflex functioning, as well as the priority of giant fiber growth relative to somatic growth, during severe and prolonged food deprivation. As a consequence of the priority of giant fiber growth during limited food availability, giant fiber conduction velocity appears to be a more reliable predictor of animal age than body size.
REFERENCES


GENERAL SUMMARY

Studies of earthworm giant fiber regeneration were made following ventral nerve cord transection, grafting, or transplantation. In addition, the effects of food deprivation on giant fiber development were examined.

In Part I, rapid and specific regeneration of medial (MGF) and lateral (LGF) giant fibers was demonstrated following VNC transection. In whole animals, through-conduction of giant fiber spikes occurred as early as 20 h, but usually by 38-48 h, after severing the VNC. The time to regeneration of the giant fibers appeared unaffected by the anterior (segment 25) versus posterior (segment 75) locus of transection in whole worms and by the absence of a head in preparations consisting of the last 40 segments of worms.

In most cases, conduction of giant fiber spikes across the transection was initially bidirectional and reliable, although in some cases conduction was intermittent and labile. Conduction in all cases was highly reliable after 4-8 h, however, reversible failure of MGF spike propagation across the transection could be evoked by repetitive stimulation (20 Hz).

In grafted preparations, MGF and LGF through-conduction was established after 65 h + 26 SD (n = 13) and 7-14 days (n = 6), respectively. Initially, conduction of the giant fiber spikes across most preparations was bidirectional and reliable, and velocities across the graft were approximately one-fifth those in normal fibers. During
the next three weeks, through-conduction remained reliable and conduction velocities increased to approximately one-half normal. The conduction properties of the regenerated giant fibers in transected and grafted preparations were consistent with previously proposed models involving electrical coupling between the severed ends of the regenerating fibers (Birse and Bittner, 1976; Balter et al., 1980; Birse and Bittner, 1981).

In Parts II and III, morphological and electrophysiological evidence was obtained for the establishment of central and peripheral connections between transplanted VNC and recipient VNC and body wall. Lengths of VNC were removed from segments 10-22 or 75-87 of one worm and transplanted into centrally denervated regions of segments 10-22 or 75-87 in a recipient animal. By 4-10 days after transplantation, the giant fibers at both ends of the transplanted VNC were joined to their counterparts in the recipient VNC and giant fiber spikes were reliably conducted across these junctions. In general, during the next 21 days giant fiber conduction velocities across the junctional region increased but never returned to normal, even after six months.

In the transplant region, the normal giant fiber morphology was gradually altered and by 4-5 weeks after transplantation, numerous branch-like extensions of the giant fibers were evident. By 9-10 months, these extensions were less abundant, many ventral cell bodies were absent, and neuropile organization was altered. Despite these morphological alterations there were no significant changes in giant fiber conduction velocity.
Peripheral function in the transplant region was also reestablished. Restoration of giant fiber sensory fields in the transplant region occurred by two weeks after surgery. Restoration of motor function in the transplant region, as indicated by MGF-mediated muscle activity, occurred within 2-3 weeks after surgery. In all cases, properties of the restored sensory fields and MGF-mediated muscle potentials in the transplant region reflected the origin of the transplanted VNC. The most likely explanation for these results was that functional restoration involved a distal regeneration of severed motor and sensory axons as well as a maintenance of sensory-to-giant and giant-to-motor pathways after transplantation.

In Part IV, the effect of food deprivation on the postembryonic development of the giant fibers was investigated. Newly hatched worms were divided into several groups, each receiving a different level of food. Giant fiber growth, as indicated by increased conduction velocities and diameters, was unaffected over a wide range of food deprivation levels whereas somatic growth, as indicated by increased body weight, was stunted by low levels of food. This result indicated a priority of giant fiber development over somatic development during food deprivation. Giant fiber growth was stunted by extreme and prolonged food deprivation but these effects were readily reversed when food was replenished.
REFERENCES


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