Target-and Stage-Dependent Influences on Axonal Outgrowth of an Identified Motorneuron in Leech CNS Explants

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TARGET- AND STAGE-DEPENDENT INFLUENCES ON AXONAL OUTGROWTH OF AN IDENTIFIED MOTORNEURON IN LEECH CNS EXPLANTS

by

April Crowley

A Thesis
Submitted to the Faculty of The Graduate College in partial fulfillment of the requirements for the Degree of Master of Science
Department of Biological Sciences

Western Michigan University
Kalamazoo, Michigan
April 2000
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April Crowley
2000
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I would like to thank several people for their assistance with this work. To begin, I would like to thank my mentor, my hero, and my intellectual papa Dr. John Jellies. His unending support and patience provided the nurturing environment I needed as a fledgling scientist, while his wit and professionalism encouraged the independence I will need to forge my own path in the scientific world.

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April Crowley
TARGET- AND STAGE-DEPENDENT INFLUENCES ON AXONAL OUTGROWTH OF AN IDENTIFIED MOTORNEURON IN LEECH CNS EXPLANTS

April Crowley, M.S.
Western Michigan University, 2000

The neural circuit that controls rhythmic contraction of the hearts in the medicinal leech, *Hirudo medicinalis*, provides an amenable model to investigate pathfinding and target selection. The bilaterally paired heart tubes (HT) in leech are eventually innervated by the heart excitor motoneuron (HE) following a period of remodeling that requires the presence of the HT. To begin characterizing possible HT-derived cues, co-cultures of CNS with innervated HT have been used. Previous work demonstrated that young, but not adult, HT was able to support profuse general outgrowth, but the HE extended few projections onto HT of any age. Current studies show that the inability of the HE to extend onto its target *in vitro* is not due to an influence of innervation state of the muscle nor is the result limited to living tissue. These results, when taken in conjunction with HE development *in vivo*, support the hypothesis that the HE is responding to a specific, target-derived signal that stops or slows extension on the target. These results suggest that the HT target tissue constitutively expresses a signal specific for the HE. Furthermore, the selective signal, as well as general growth-permissive substrates, survives in the matrix despite the loss of intact, viable muscle cells.
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INTRODUCTION

Literature Review

The generation of neural circuits during development is often seen as a very precise process involving several factors including pathfinding, trophic and tropic influences, and complex cell-cell interactions. Many of these developmental influences have been described in vertebrate and invertebrate models (Goodman and Shatz, 1993; Frank and Wenner, 1993; Jellies and Johansen, 1995) supporting the view that similar mechanisms are used in various species to guide the development of neural circuits. For instance, guidepost or landmark cells have been described in both grasshopper (O’Connor et al., 1990) and zebrafish (Kuwada and Bernhardt, 1990; Greenspoon et al., 1995); similar contact-mediated cues or cell adhesion molecules have been reported in grasshopper (Raper et al., 1983; Goodman et al., 1984) and chick models (Bonhoeffer and Huf, 1980); examples of pioneer neurons may be found in grasshopper (Klose and Bentley, 1989), fish (Kuwada, 1986), and mammal (McConnell et al., 1989).

Growth cones are able to respond to environmental cues in specific and distinct ways allowing for the development of stereotypical neural pathways and synaptic connections. Some of these pathways appear to be relatively nonselective in that they provide a permissive substrate for general axonal growth while at the same
time may also contain specific cues to which only a subset of growth cones are able to respond (Lance-Jones and Landmesser, 1981; Tosney and Landmesser, 1985). In embryonic chick, axons were able to locate and innervate their appropriate target when that target had been displaced by two or three segments anterior-posterior (a-p). Displacement by a greater distance resulted in a failure of the extending motoneuron to form stereotyped connections (Lance-Jones and Landmesser, 1981). It has been suggested, based on these studies, that specific cues acting on a subset of the growth cone population may be local, perhaps existing as trophic influences and allowing for altered growth rate and/or stabilization of growth cone filopodia (Lance-Jones and Landmesser, 1981). Once the extending axon has reached the region of its future postsynaptic target, local interactions between the presynaptic growth cone and its eventual target dominate synaptic development. This cell-cell interaction involves anterograde communication, in which signals are passed from the presynaptic terminus to the target cell, and retrograde communication, where signals are transferred to the growth cone from the target (Goodman and Shatz, 1993; Haydon and Zoran, 1994).

Examples of such communication between the developing presynaptic process and its postsynaptic target are found in many vertebrate and invertebrate models (reviewed by Frank and Wenner, 1993; Goodman and Shantz, 1993). For example, in *Helisoma*, studies examined synapse formation in the B19 motoneuron. When contact was made between the B19 motoneuron and inappropriate targets, action potentials failed to provoke the release of neurotransmitter although calcium channels
were found to be present in neurites contacting the new target. Increased calcium was able to induce neurotransmitter release only after the neurites were in contact with the correct target muscle for several hours. This shows that the ability of the B19 neuron to release neurotransmitter is gained in a target-dependent manner. It is suggested that the postsynaptic target is able to send instructive retrograde signals, via cell adhesion molecules or growth promoting factors, to the presynaptic growth cone (Haydon and Zoran, 1994).

The leech, *Hirudo medicinalis*, provides many examples of cell-cell interactions driving neurodevelopment. The first documented example of peripheral influence over the development of identified neuronal phenotype involved the Retzius neuron (Rz). Retzius neurons are neuromodulatory cells found in most segments of the leech that project four ipsilateral axons, two extend into the periphery of the segment of origin while two extend into adjacent segments (one rostral and one caudal) to project into the periphery (Glover and Mason, 1986; Jellies et al., 1987). In the periphery, these neurons innervate a large area of muscle and skin, functioning as modulators of muscle tension and mucus secretion (Lent, 1973; Mason and Kristan, 1982).

The pattern of Rz development and function is identical in all but two segments. Segments five and six of the leech midbody contain the sexual organs of the animal which are innervated by the Rz cells (Rz5,6) of those segments (Glover and Mason, 1986; Jellies et al., 1987). Rz5,6 do not project axons to the body wall nor do they extend axons into adjacent segments (Glover and Mason, 1986; Jellies et
Initially during development, however, all Rz cells, including those in segments five and six, extend four axons in the pattern mentioned previously (Glover and Mason, 1986; Jellies et al., 1987). Once the growth cones are in the area of the developing sex organs, several changes in the neuronal phenotype begin including an alteration in growth cone size and shape, termination and retraction of projections into the intersegmental connectives (Glover and Mason, 1986; Jellies et al., 1987), and termination of growth cone extension into the body wall (Jellies et al., 1987).

Removal of the sex organs results in Rz5,6 that have a phenotype similar to that found in other segments, i.e. lateral body wall projections and interganglionic extensions (Loer et al., 1987; Macagno et al., 1986). This result indicates that the developmental changes in Rz5,6 are not simply coincidental. These phenotypic changes in Rz5,6 are driven by a retrograde influence received from the sex organ primordia (French et al., 1992), but the identity and extent of action of any such cue in any experimental system has remained obscure.

Yet another example of cellular communication found in leech neurodevelopment can be found in the rostral penile evertor motoneurons (RPEs). In the adult animal, the RPEs innervate the male sex organs. During development, the RPEs extend multiple branches. Following contact and innervation of the target by two of these branches, the remaining branches are withdrawn. Experiments investigating target ablation resulted in the retention and continued growth of supernumerary branches. When target tissue was transplanted to foreign locations, RPE branches were able to locate and innervate the ectopic target (Baptista and Macagno, 1988).
These studies demonstrate the significance of target-derived signals in the acquisition of appropriate neural phenotypes.

The ability of a developing neuron to navigate correctly through a continuously changing environment, locating its suitable target, has been the focus of a great deal of research. It is hoped that by identifying the cues involved in the correct development of neural circuits we may begin to address the issues in many neurodegenerative diseases. Work in this thesis involves investigating aspects of an ongoing project examining the development of a highly defined circuit, that which is involved in the neural control of the hearts in leech, *H. medicinalis*.

The System

The leech is an excellent model for the study of neural circuit development at the cellular level. The central nervous system (CNS) consists of 32 segmental ganglia, the first four are fused to form the head region of the animal, the last seven form the tail region, and the remaining 21 ganglia are known as the midbody ganglia and are numbered 1-21 (Figure 1). Each ganglion houses approximately 200 bilaterally paired neurons, many of which have been uniquely identified (Figure 2) (Muller et al., 1981). Ganglia in each segment are similar with the exception of those found in segments five and six. Being hermaphroditic, leeches possess both male and female sex organs which reside in segments five and six respectively. The ganglia of these segments contain more neurons (approximately 700) and are considered more specialized than the others.
Figure 1. Diagrammatic Representation of the Central Nervous System and Heart Tubes in Leech. This diagram shows the location of the lateral heart tubes in relation to the ganglia of the central nervous system. The ganglia are enclosed in a ventral blood sinus. Also present but not depicted is a dorsal blood sinus. Shaded areas of the heart tubes indicate the regions receiving innervation from heart excitor motorneurons, unshaded portions represent area where the heart tubes fuse with the ventral and dorsal sinuses. Figure is positioned with anterior upward.
Figure 2. Ventral View of a Mid-Body Ganglion. The bilaterally paired HE cells can be identified by their position with respect to other neurons. Dashed lines indicate packet boundaries within the ganglion. Each packet is defined by a single glial cell. The HE cells reside in the anterolateral packet of the ganglion and project their axons out the ipsilateral anterior nerve roots. They are the only neurons in this quadrant to follow this pathway out of the ganglion which also aides in their identification. The anterior nerve root is indicated by A, the posterior nerve root is indicated by P. Ganglion is drawn with anterior upward.

The circulatory system of the leech is a closed system consisting of two lateral vessels known as the heart tubes, a dorsal sinus, and a ventral sinus, which surrounds the CNS. These four major vessels are joined at the front and rear of the animal forming a continuous closed system (Figure 1). In each segment, there are vessels and capillaries present, which cross-link the major longitudinal sinuses. Blood is pushed forward by the pumping action of the heart tubes and allowed to flow rearward through the ventral and dorsal vessels (Thompson and Stent, 1976; Stent et al., 1979).
The coordinated pumping action of the heart tubes is imposed by the heart excitor motorneurons (HE) (Thompson and Stent, 1976; Maranto and Calabrese, 1984a). The HE motorneurons are bilaterally paired within the ganglia, both their position and their morphology have been well characterized and are consistent from segment to segment (Figure 2). These functionally-defined motorneurons are found only in segments 3 through 18; however, probable homologs have been documented in the first two and last three segments (Jellies et al., 1992). The heart tubes themselves exhibit myogenic activity (Thompson and Stent, 1976; Stent et al., 1979; Maranto and Calabrese, 1984b), but their rhythmic contractions are the result of a set of heart interneurons known as HNs which interrupt the tonic activity of the HE. The HN cells are found in ganglia 1-7 and form inhibitory synapses with the HE cells. It is this phasic inhibitory input from the central pattern generator (CPG) to the HE which causes the rhythmic contraction of the hearts (Thompson and Stent, 1976; Stent et al., 1979; Tolbert and Calabrese, 1985). Thus, the functional heart circuit is relatively simple, being composed of a muscle target, one motorneuron, and one CPG input.

Experimental Background

In studies examining the specificity of heart tube innervation by the HE, Dil injections of the heart tube lumen were performed. Dil is a lipophilic dye which is transported (with low probability) through some of the synapses of neuromuscular junctions; therefore, neurons which form membrane appositions should be labeled. This technique has shown that only the HE innervates the heart tube and that the
mature HE has no other target but the heart tube (Jellies, 1995; Harik, 1998; Harik et al., 1999).

Using intracellular dye-filling and antibody staining, it was found that, early in development, the HE extends elaborate branches to both the expected area of the nascent heart tube and additional arbors in the body wall. As the heart tube begins to develop (embryonic day 12 (E12)), the arborizations in the vicinity of the heart tube leave the body wall and expand on the HT while those in the body wall are retracted. This remodeling suggests that early growth may be in response to pathway cues for growth cone migration while the HE-heart tube interaction represents a specific local interaction (Jellies et al., 1992). Studies examining this HE-heart tube interaction tested the hypothesis that the developing heart provides cues involved in target selection and final differentiation of the HE motor neurons. Again, intracellular dye-filling and immunocytochemistry were employed in addition to ablation of the heart primordium. Target-deprived HEs continue to project axonal branches into the body wall in a manner resembling that seen during the early embryonic stages. Additionally, the generation of the functional circuit is compromised in the absence of each HE-heart contact. This suggests that HE development requires contact with the developing heart tube (Jellies and Kopp, 1995). Thus, cues associated with the target HT appear to play a defining role in the assembly of this circuit.

A co-culture system was developed to investigate the ability of heart-derived cues to stimulate axon outgrowth in vitro, as well as examine the notion that these cues are stage-dependent. In the system, adult ganglion explants were co-cultured
with either body wall or heart tube taken from the embryonic, juvenile, or adult stage of leech development. The results showed that embryonic, as well as juvenile, body wall and heart tube are able to stimulate profuse outgrowth from the adult CNS onto these tissues as well as onto the perineural sheath. Adult body wall and heart tube, however, support very little outgrowth. Adult axons, in all cases, were able to grow onto the perineural sheath, indicating an ability to send extensions (Harik, 1998; Harik et al., 1999).

Preliminary studies to identify the axons responsible for the outgrowth seen in vitro involved intracellular filling of HE cells with fluorescent dextran prior to culturing with embryonic heart tube. This method was used to test the prediction that the HE was exhibiting outgrowth onto the heart tube. However, contrary to this naïve prediction, the HE does not extend elaborate processes onto the heart tube under in vitro conditions (Harik et al., 1999). Rather than being a simple negative result, the great specificity with which the HE does not contribute axons under experimental conditions provides a potent tool to investigate the target interactions involved.

The experiments in this thesis expanded upon an established co-culture design to test the possibility that the target presents highly selective cues that slow or stop HE outgrowth. This might allow for the formation of stable synapses between the HE and the HT or induce lateral branches.
MATERIALS AND METHODS

Animals

Embryonic and juvenile animals were obtained from a breeding colony at Western Michigan University as described previously (Jellies et al., 1987), maintained at room temperature and development staged at 22-25°C (Fernandez and Stent, 1982; Reynolds, et al., 1998). External features of the developing animal are used in staging. Pumping of the larval mouth terminates during embryonic day 9 (E9), or at approximately 45.5% embryonic development (ED). A shadow of a line separating the posterior 7 segments, which will form the posterior sucker, is visible at E10 or 47.5% ED. At E11, or approximately 50.5% ED, this line becomes more pronounced and the edges of the posterior sucker have begun to rise, but have not yet fused, which gives a horseshoe appearance. During E12, 52.5% ED, the posterior sucker becomes fused. The appearance of a white line over the first four segments, indicating dorsal fusion of the germinal plate, is observed on day 16 of embryogenesis. Completion of this fusion, as evidenced by a white line down the entire dorsal midline, occurs on embryonic day 20, or at 68.5% ED. Embryogenesis is completed at E30. Embryonic tissue used in these studies was obtained from animals between the stages of 47.5% and 93.5% ED. Juvenile animals used as
sources of tissue were postembryonic 1-31 days and had not been fed. Adult animals were obtained from the Western Michigan University breeding colony and Leeches USA and were known to be at least 1 year in age or 2-3g in weight.

Prior to dissection, adult animals were immobilized in cold Ringers solution (Muller et al., 1981) for 20-30 minutes. Embryonic and juvenile animals were anesthetized with 8% ethanol in sterile saline (Jellies et al., 1987).

Intracellular Dye Injections

Segments of nerve cord were removed from adult animals and pinned ventral side up in saline-filled culture dishes coated with sylgard. Cells within the ganglia were visualized with the use of dark-field illumination and impaled with microelectrodes made from thin walled borosilicate glass capillaries. Electrical activity was used to confirm cell identity. Cells were filled with Texas Red- or biotin-conjugated dextran (2-5% weight/volume in 0.2 M KCl; 3000 MW, Molecular Probes) by passing 3-5nA of depolarizing current for 5-15 minutes. Cells filled with biotin-conjugated dextran were further processed with Texas Red-, Fluoroscein-(FITC), or horseradish peroxidase- (HRP) linked streptavidin (Harik et al., 1999).

To fill embryonic neurons, animals approximately 47.5-50.5% ED were anesthetized with 8% ethanol/sterile saline. An incision along the entirety of the dorsal midline was made, the animal pinned open, and yolk material removed. The embryo was then repinned ventral side up and lengthwise slits cut through the germinal plate over groups of three ganglia. Neurons were then impaled with
microelectrodes and filled with a fluorescent dye (Jellies et al., 1992). Texas Red-conjugated dextran was injected using 1-2 nA depolarizing current until the cell was visible under ultraviolet light.

**Synaptically Naïve Heart Tube**

Animals at 47.5% ED were anesthetized in 10% ethanol/embryo water (Jellies et al., 1987) and held in cavities cut in sylgard coated petri dishes. Chains of six or more ganglia were removed by making small incisions in the germinal plate over two separate ganglia. The connective of a posterior ganglion was snipped and the nerve cord removed by grasping the anterior ganglion with fine forceps and tugging. The embryos were then rinsed twice in embryo water and placed into individual dishes for further development (Jellies et al., 1995).

**Heart Tube Explants**

Animals between the stages of 62.5% ED and P31 were used as heart tube donors. The embryos were anesthetized in 8% ethanol/sterile saline and cut along the dorsal midline along their entire length. Following pinning, yolk material was removed. Using microdissection tools (sterilized in 95% ethanol and rinsed in sterile saline), gut and muscle tissues were peeled off the heart tubes. Sections of heart approximately .5 to 1 segment in length were then removed and used in culture (Harik, 1998; Harik et al., 1999).
For generation of dead HT, the entire heart on either side was removed from donor animals between the stages of 62.5% ED and 70% ED. The HT was then pinned in a saline-filled culture dish coated with sylgard, and frozen and thawed repeatedly, applying fresh saline each time. This process tends to break and remove membranes. The dead HT was then kept at 4°C for up to 3 days prior to use.

Tissue Culture

Tissue explants were plated on poly-L-lysine (Sigma) coated glass coverslips (Assistant Inc.) in 35 mm sterile culture dishes (Falcon) containing approximately 2 ml sterile culture medium. The medium consisted of Leibovitz L-15 medium (Sigma) diluted 25% with sterile saline and supplemented with 6 mg glucose/ml (Sigma) and 0.06 mg glutamine/ml. Penicillin-streptomycin (5 mg/ml; liquid, 5000 U, Sigma) was added to obtain a final dilution of 10%. The anterior nerve roots of the ganglion were cut long, proximal to the first bifurcation, while the posterior nerve roots were cut short, close to the ganglionic margin. Both the anterior and posterior connectives were cut approximately midway between two ganglia. Each ganglion was plated and the nerve roots arranged with sterile glass micropipets. Segments of heart tube were arranged so as to be in contact with the cut end of the anterior nerve root of the ganglion (Figure 3) (Harik, 1998; Harik et al., 1999).

For cultures with explanted embryonic nerve cord, chains of three ganglia were removed by snipping the connectives at either end, grasping the loose connective, and pulling the chain out without first snipping the nerve roots. Ganglia
Figure 3. Schematic Drawing of the Co-Culture Design. Single adult ganglion with prelabeled (Texas Red- or biotin-dextran) HE cells were plated on poly-L-lysine coated coverslips. Segments of embryonic HT were placed in contact with the cut end of the anterior nerve root. Cultures were incubated at room temperature for 5 days in supplemented L-15 culture medium prior to fixation and antibody processing.

were then plated on poly-L-lysine coated coverslips with sections of heart tube approximately 3 segments in length. The heart tube was placed in contact with the ganglionic margin. Culture medium used was supplemented L-15, as described previously.

In both situations, 30-40 µl of an equal mixture of goat serum (Sigma) and ITS liquid supplement (Sigma) was sterile filtered (Acrodisc, Gelman Sciences) into each dish after 24 hours. Cultures were then left for 5-6 days in a laminar flow hood at room temperature after which they were processed for immunocytochemistry.

In this study, four different experimental situations were examined: (1) co-culture of adult ganglion with innervated young heart tube, (2) co-culture of adult
ganglion with naïve young heart tube, (3) co-culture of adult ganglion with young, innervated, dead heart tube; and (4) co-culture of embryonic ganglia with innervated embryonic heart tube.

Immunocytochemistry

Cultured tissues were fixed overnight with 4% paraformaldehyde (Spectrum) in 0.1M phosphate buffer (pH 7.4). Following fixation, preparations were extensively rinsed with PBS. A monoclonal antibody to acetylated tubulin (ACT; Sigma) was utilized to identify neuronal projections (Jellies et al., 1995; 1996). Prior to application, the antibody was diluted 1:1000 in PBS with 10% goat serum, 1% Triton X-100, and 0.001% sodium azide. Incubations were carried out overnight at 4°C. Tissues were extensively rinsed with PBS prior to the addition of the secondary antibody. Texas Red-conjugated (at 1:500; Molecular Probes) or FITC-conjugated (at 1:50 to 1:250; Molecular Probes or Jackson) goat anti-mouse IgG was diluted in the same buffer described above and again allowed to incubate overnight at 4°C. In preparations with neurons filled with biotin-conjugated dextran, an incubation with either Texas Red-streptavidin (at 1:500; Vector) or FITC-streptavidin (at 1:250; Vector) was performed overnight in the refrigerator. The streptavidin conjugates were diluted in PBS buffer with 1% Triton X-100 and 0.001% sodium azide. Additionally, following a brief rinse with Tris, preparations were incubated for 13 minutes at 4°C with bis-benzimide (Sigma) 0.01mg/ml in 0.1M Tris to label all nuclei. This label allowed the visualization of the otherwise transparent heart tube.
and also served to confirm the health of the explanted tissues. Tissues were mounted in glycerol and visualized using an epifluorescent Nikon microscope with appropriate filter sets (Harik, 1998; Harik et al., 1999).

In another set of preparations, biotin-conjugated dextran was used to fill neurons and the tissues subsequently incubated with HRP-streptavidin (Vector) 1:500 in PBS with 1% Triton X-100 and 0.001% sodium azide overnight in the refrigerator. The preparations were rinsed repeatedly with PBS followed by Tris. The tissues were then incubated with 3,3’-Diaminobenzidine tetrahydrochloride (DAB) (.5 mg/ml Tris; Gibco) for 15 minutes at 4°C after which, 2% CoCl₂ was applied for approximately 2 minutes. Reaction with H₂O₂ was stopped with cold Tris once a black precipitate was visible. The preparations were then rinsed extensively in Tris, dehydrated in ethanol, cleared in methyl salicylate, and mounted in Cytoseal 60. HRP labeled mounts were visualized using DIC optics.

Data Analysis

These data were analyzed and presented by several means. The primary, and most basic, method was visual interpretation. Preparations were viewed and analyzed by comparing, visually, outgrowth patterns. In many cases this is the preferred and most direct analysis. While not quantitative, ample precedent exists in the literature (F. de-Miguel and Vargas, 1997; Wang et al., 1999; Skutella et al., 1999) for qualitative assessment, particularly when responses are all-or-none. Additionally, HRP-labeled processes were examined and outgrowth quantified by counting both the
number of varicosities and processes extending from the HE onto the surface of the HT further than 20 μm (Figure 4). HRP-labeled varicosities and processes that remained at the nerve terminal or grew back along the perineuronal sheath were counted separately. Values for the HRP data are presented as number of varicosities, number of processes, and as density which was calculated as the number of varicosities per process. To determine whether variation was associated with experimental condition, HE outgrowth onto the HT was normalized to the total HE outgrowth. This comparison is necessary to account for variation in general outgrowth and sprouting. Statistical analysis of the values obtained consisted of the T test using a significance level of p<0.05.

Figure 4. Quantification of HE Outgrowth Using HRP. HEs were labeled by biotin-dextran injection followed by HRP processing. The labeled HE varicosities (arrowheads) and processes (arrows) were then counted and used for quantitative analysis of HE outgrowth onto target tissue.
RESULTS

The HE Exhibits Limited Outgrowth Onto Young Heart Tube In Vitro

In order to confirm the previous observation that the HE motoneuron extends poorly upon its specific target in vitro, I repeated prior co-culture experiments (Harik, 1998; Harik et al., 1999). In these experiments, an adult ganglion with HEs prelabeled with Texas Red-dextran was cultured in contact with young innervated HT. I was able to label successfully 25 HE motoneurons in 13 ganglia. Of these 25 prelabeled HEs, 21 had axons in the anterior nerve root (ANR) which were in contact with the young HT. One of the HEs lacked any varicosities or branching, but seemed otherwise healthy. Only two HEs exhibited robust outgrowth, displaying multiple varicosities and branches onto the target tissue. In the remaining 18 examples, outgrowth was limited to very short extensions onto the HT which terminated immediately in varicosities or projection of a few small sprouts onto the HT (Figure 5). All adult axons, including the HE, retained the ability to extend; however, the HE accounted for little of the outgrowth. Most of the outgrowth displayed by the HE was back along the neural sheath. In instances where outgrowth of the HE onto the HT was seen, it was accompanied by profuse general outgrowth (as viewed by ACT staining). This result is identical to previous work on this system (Harik et al., 1999).
Figure 5. The HE Exhibits Limited Outgrowth Onto Target Muscle In Vitro. Adult ganglion with prelabeled (FITC) HEs was cultured in contact with embryonic HT. General axonal outgrowth was labeled using an antibody to acetylated tubulin (ACT) followed by a Texas Red-linked secondary antibody (Red). Adult axons retain the ability to grow, however, the HE accounts for a relatively minor portion of the outgrowth seen as demonstrated by comparison of B (just the green channel) and C (just the red channel).

Fluorescent Dextran Label Does Not Inhibit HE Outgrowth

While the discovery that the HE does not extend significantly onto its target tissue in vitro was certainly interesting, I wondered if perhaps this was the result of a
negative influence by the fluorescent label. This possibility does not seem likely (Harik et al., 1999). To begin with, the central anatomy of the prelabeled cells appeared normal. Dendritic and axonal processes appeared normal in that they had a predicted branching pattern and the processes themselves were smooth and continuous. Also, the HE was able to extend several sprouts, if not onto the HT, then back into the nerve root or onto the nerve sheath. In addition to experiments using ganglia with prelabeled HEs, several co-cultures were done as previously described, but without prelabeling HEs. In these preparations, the cover slips were removed from the culture medium following 5 days incubation and placed in a recording chamber containing saline. The HE cells were then filled with fluorescent dextran, fixed after approximately 2 hours at room temperature, and processed as previously described (Materials and Methods). Of the 4 HE neurons (in 3 ganglia) completely filled with dye and in contact with the embryonic HT, all were limited in the amount of outgrowth displayed. This result was consistent with the results obtained by prelabeling HEs (Harik et al., 1999).

Experiments using a non-fluorescent dextran dye were also performed. In these studies, HE motoneurons were injected intracellularly with biotin-dextran, cultured for 5-6 days and processed with fluorescent or HRP-linked streptavidin for HE labeling. Twenty-five HEs in 17 ganglia were filled successfully and of these, 16 had axons in an ANR that was touching the young HT. One of the 16 HEs failed to grow out of the ANR, either onto the neuronal sheath or onto the HT, and failed to form varicosities. Five HEs were able to extend multiple branches and varicosities
onto the explanted HT. The remaining 9 HEs displayed limited growth in that they formed varicosities immediately upon exiting the ANR or extended just a couple small sprouts to the HT. Again, the results were consistent with prelabeling with fluorescent dextran.

Is There an Innervation-Dependent Target Selection Cue?

Having eliminated several simple explanations for the inability of the HE to extend lavishly onto the HT, I was left with a more complex possibility. I thought it possible that the innervated HT we had been using was expressing an HE-specific signal which deterred further elaboration by the HE, or that innervation had altered HT expression of a growth promoting factor. To test these ideas, I decided to repeat the cultures using synaptically naïve HT. Large contiguous portions (6-16 segments) of the HT donors’ CNS were surgically removed at 47.5% ED (as described in Materials and Methods), prior to the emergence of the HT. This provided contiguous segments of synaptically naïve HT. Since it seemed entirely possible that the HE from the innervated segments might extend into the naïve regions of the HT, the denervation procedure was confirmed before continuing with the cultures. To confirm the naivete of the HT, several animals were dissected and processed with ACT (as described previously). These examinations showed that the HE innervates its segment of origin as well as half of one segment anterior and posterior, leaving segments of denervated HT (Clark et al., 1999a; 1999b).
Naïve HT Does Not Stimulate Increased HE Outgrowth

If HT was unable to produce a signal encouraging HE extension as a result of prior innervation, one might predict that synaptically naïve HT would elicit profuse outgrowth from the HE. To test this prediction, the culture experiments were repeated using synaptically naïve HT. As before, the HE motorneurons were prelabeled with Texas Red-dextran or biotin-dextran and processed for fluorescence or HRP staining as described previously. Ninety-eight HEs were labeled in 59 ganglia. Of these 98, 71 were visible, without damage, and in an ANR contacting the young, naïve HT. Only 7 HEs had processes extending further than 20µm along the HT. In this set of experiments, 3 HEs failed to demonstrate any type of growth. Surprisingly, only 10 HEs displayed robust outgrowth onto the explanted HT, extending multiple branches and varicosities; in many of these preparations, the elaborate HE outgrowth was accompanied by profuse axonal outgrowth in general. Again, the majority of preparations (51) exhibited outgrowth which was limited to extending a few small sprouts or terminating immediately in varicosities (Figure 6) (Table 1). For control experiments, pieces of innervated HT were used. Of 104 successfully filled HEs in 61 ganglia, 81 had axons in ANR that were in direct contact with the target tissue. Of these 81, 8 were able to extend processes onto the HT longer than 20 µm. Only 12 examples of the HE sending multiple branches and/or varicosities onto the HT were obtained. Again, 3 HEs failed to grow onto the HT or the nerve sheath, or to produce any varicosities, although the cell appeared otherwise
Figure 6. Synaptically Naïve HT Does Not Stimulate Increased HE Outgrowth. HE outgrowth onto naïve target was equal to, but no greater than, outgrowth onto innervated target. HEs were prelabeled with Texas Red and general outgrowth identified using ACT followed by a FITC-conjugated secondary antibody. Outgrowth by the HE onto naïve HT is characterized by immediate formation of varicosities (arrowheads) and the occasional small sprout (arrows) (A). This is comparable to outgrowth patterns seen in cultures using innervated HT (B). Images are also presented in only the red (A’ and B’) or green (A” and B”’) channels.

healthy. The remaining 58 HEs in the proximity of explanted HT had outgrowth that was modest (Figure 6) (Table 1). Once again, they formed bulbs at the location of contact or extended a few small caliber sprouts onto the HT. In most cases, the
outgrowth displayed by the HE was back onto the nerve sheath (Crowley et al., 1999a; 1999b). Thus, HE outgrowth onto the naïve target was qualitatively comparable to that seen with innervated target.

Table 1

<table>
<thead>
<tr>
<th>Heart tissue type</th>
<th>Number of ganglia</th>
<th>Number of labeled HEs</th>
<th>HEs contacting HT</th>
<th>HEs without robust outgrowth</th>
<th>HEs with &gt;20mm</th>
<th>HEs with robust outgrowth &gt;20mm</th>
<th>HEs with modest outgrowth</th>
</tr>
</thead>
<tbody>
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<td>Naïve</td>
<td>59</td>
<td>98</td>
<td>71</td>
<td>3</td>
<td>10</td>
<td>7</td>
<td>51</td>
</tr>
<tr>
<td>Innervated</td>
<td>61</td>
<td>104</td>
<td>81</td>
<td>3</td>
<td>12</td>
<td>8</td>
<td>58</td>
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<tr>
<td>Dead</td>
<td>27</td>
<td>34</td>
<td>21</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>17</td>
</tr>
</tbody>
</table>

Outgrowth patterns of the HE onto naïve, innervated, and dead HT in vitro were described qualitatively. Visual inspection of the preparations revealed no difference in amount or patterning of outgrowth onto the different heart tissue types.

Quantitative assessment of the data strongly supports what has been described qualitatively. Analysis of varicosity and process number in HRP-labeled preparations show no difference between outgrowth onto naïve or innervated HT (p < 0.05) (Figure 7). Mean varicosity number for innervated HT was 5.52 +/-1.46 S.E.M. as compared to 7.83 +/-2.94 S.E.M. for naïve HT. Mean process number for innervated HT was 2.13 +/-0.46 S.E.M. as compared to 2.29 +/-0.63 S.E.M. for naïve HT. Finally, varicosity density onto innervated and naïve tissue also revealed no difference (1.57 +/-0.34 S.E.M. and 1.82 +/-0.41 S.E.M., respectively; p < 0.05).
Figure 7. Outgrowth Patterns Onto Innervated and Naïve HT Are Similar. The number of varicosities (A) and processes (B) extending >20µm onto the target tissue were counted. Varicosity density (C) was calculated as v/p. No significant difference was detected between outgrowth onto innervated or naïve HT. Error Bars represent +/- S.E.M.
Because there seemed to be substantial variation in axonal outgrowth (some HEs sprouted in general much more than others) I wanted to normalize outgrowth onto the HT to total outgrowth by the HE for each preparation. For this analysis, varicosity number and process number on both the HT and nerve sheath were counted. The number observed onto the HT was then divided by the total. These normalized values were used to compare the amount of outgrowth onto naïve and innervated target tissues. No difference in either varicosity index or process index was detected between naïve and innervated HT ($p < 0.05$) (Figure 8). Mean varicosity index for innervated tissue was $0.274 +/- 0.058$ S.E.M. as compared to $0.297 +/- 0.063$ S.E.M. for naïve tissue. Mean process index for innervated and naïve tissues was $0.235 +/- 0.047$ S.E.M. and $0.235 +/- 0.052$ S.E.M., respectively. It should be noted that the formation of varicosities does not equate to synapse formation. The appearance of the bulbous varicosities on regenerating neurites is a common occurrence which is used to characterize neuronal outgrowth (F. de-Miguel, 1997).

**The HE Does Not Elaborate Extensively Onto Dead HT**

Preliminary work was begun on the characterization of a possible negative regulator of HE outgrowth expressed by the young HT. It has been suggested that such a cue may function to slow growth of HE processes and thereby promote formation of stable synapses between the HE and its appropriate target, the HT. One potential regulator that has been described (Biswas *et al.*, 1999) is a receptor tyrosine phosphatase. This membrane-bound receptor has been found in living HT muscle
Figure 8. Normalization Confirms Outgrowth Similarities Between Innervated and Naïve HT. Normalization of varicosity (A) or process number (B) to total exhibited on both neural sheath and HT reestablishes the likeness in outgrowth onto innervated and naïve tissue. Error Bars represent +/- S.E.M.

cells. Subjecting pieces of HT to repeated freeze/thaw cycles serves to kill the tissue, preventing production of potential signaling proteins, while leaving the three dimensional structure of many surface molecules and substantial basal lamina intact (Merz and Drapeau, 1994). If the HE-specific cue is indeed present in the matrix of the HT, functioning in a contact-mediated fashion, the growth cone of the HE would still be able to recognize the molecule and respond to it appropriately, i.e. exhibiting limited outgrowth onto the HT. If, on the other hand, the negative signal is a diffusible factor or a product regulated by phosphatase activity, the dead tissue would lack it and the HE would be expected to elaborate extensively onto permissive (Harik,
1999) matrix of the HT. To test this idea, co-culture experiments using dead HT and adult ganglion were performed. As before, the HE cells were prelabeled with biotin-dextran and processed for fluorescence or HRP staining as described previously. I was able to label successfully 34 HE motoneurons in 27 ganglia. Of these 34, 21 had axons in an ANR which was in contact with the dead HT. Only one of the HEs failed to form varicosities or extend any processes either to the nerve sheath or onto the HT although the cell otherwise appeared healthy. Two of the HEs exhibited more lavish outgrowth onto the HT than typically seen, extending multiple varicosities and/or processes. The remaining 17 HEs again exhibited outgrowth characteristic of that seen in the other experimental situations, formation of varicosities immediately at the cut end of the ANR, and/or 1-2 short sprouts onto the HT (Figure 9) (Table 1). Interestingly, in two of the preparations without contact between the ANR and the HT, the HE was able to travel the distance to make contact with, and display impressive outgrowth onto, the dead HT.

Figure 9. HE Outgrowth Onto Dead Heart Tissue Is Limited. Outgrowth of HRP labeled HEs is characterized by an occasional small caliber sprout (arrows) and the formation of immediate varicosities (A). Outgrowth onto dead, innervated tissue (B) was no better than that seen with living tissue, presenting few varicosities or sprouts (arrow).
Quantitative analysis of the outgrowth exhibited onto dead, innervated heart and onto living, innervated heart was performed. The number of varicosities, the number of processes, and the varicosity density seen in dead HT (4.25 +/-1.92 S.E.M., 1.94 +/-0.74 S.E.M., and 0.91 +/-0.31 S.E.M., respectively) was not different from that seen in living HT (5.52 +/-1.46 S.E.M., 2.13 +/-0.46 S.E.M., and 1.57 +/-0.34 S.E.M., respectively; p < 0.05) (Figure 10).

Again, due to significant variation in general sprouting by the HE, process and varicosity formation onto HT was normalized to the total amount of HE outgrowth seen. Comparison of varicosity index for innervated and dead HT (0.274 +/-0.058 S.E.M. and 0.250 +/-0.082 S.E.M., respectively) shows no difference (p <0.05) (Figure 11). Process index comparisons again reflect no difference between innervated and dead target tissue (0.235 +/-0.047 S.E.M. and 0.234 +/-0.074 S.E.M., respectively) (Figure 11).

Embryonic Neurons Respond Poorly to Culture Conditions

If the explanation for the HE’s inability to grow onto its normal target tissue in vitro cannot not be found in the naivete of the HT, I thought that perhaps the answer lies in the neuron itself. It could be possible that some retrograde signal following synapse formation alters the ability of the mature neuron to grow onto the explanted HT, or CNS connections irreversibly induce changes in the motoneuron. In other words, the adult neurons are not naïve either and it is known that they are altered by a retrograde signal from the HT (Jellies and Kopp, 1995). To investigate
Figure 10. HE Outgrowth Onto Dead Tissue Is Limited. Outgrowth patterns onto dead heart tissue was similar to that seen on living heart tissue as no difference was detected in varicosity number (A), process number (B), or varicosity density (C). Error Bars represent +/- S.E.M..
Figure 11. Normalization Reaffirms the Limited Outgrowth Onto Dead HT. Number of varicosities (A) or processes (B) extended by the HE onto the HT were normalized to the total number onto the sheath and HT. Such analysis confirms the similarities in outgrowth levels onto innervated living HT and innervated dead HT. Error Bars represent +/- S.E.M..

this possibility, embryonic HEs were prelabeled with Texas Red-conjugated dextran on day 10 of development, prior to HT formation. The ganglia were then cultured alone or in contact with innervated embryonic HT as described previously.

Surprisingly, the embryonic neurons failed to extend processes under the culture conditions used. Examination of injected HEs revealed healthy nuclei and intact dendrites. Although the HE was not yet dead, the axon appeared discontinuous before leaving the ganglion and showed no evidence of outgrowth. General axonal labeling with ACT also showed few neurons within the ganglion were expressing
acetylated tubulin, associated with actively growing axons in leech (Jellies, 1995; 1996), and there were also many discontinuous axons within the connectives (Figure 12). Repeated experiments (n=28) showed this result was not due to experimental technique. The same result was achieved with both refrigerated and non-refrigerated tissues, FITC and Texas Red labels, and isolated tissue culture and co-culture. Furthermore, adult nerve cord used in control experiments appeared healthy with good ACT staining and process extension onto the nerve sheath. Thus, these intriguing results suggest that embryonic neurons are dependent upon an unidentified trophic influence for growth. However, until that influence has been identified, comparable in vitro experiments cannot be performed.
Figure 12. Embryonic Neurons Respond Poorly to Culture Design. Portions of embryonic CNS were cultured in conditions identical to those used for adult CNS. Unlike adult neurons (C), embryonic neurons appeared to lose the ability to grow once removed from the animal. Processing cultured tissue with ACT followed by a FITC linked secondary antibody labeled few axons and many that were labeled lacked evidence of outgrowth and appeared discontinuous (A and B).
DISCUSSION

Work in this thesis extends an ongoing project that focuses on identifying how spatial and temporal expression of target-associated cues orchestrates circuit formation. The highly defined neural circuit that controls the hearts in the leech, *H. medicinalis*, was chosen as a model system due to the accessibility and simplicity of the nervous system. Such a system allowed me to examine the presence of target-derived signals that influence innervation of the target muscle and also allowed me to begin characterization of these signals. Specifically, I was interested in determining whether the innervation state of the target tissue has an influence on the cues that are presented to growing axons. I predicted that naïve tissue, as opposed to previously innervated tissue, would be better able to stimulate/support outgrowth from its normal synaptic partner, the HE motoneuron. This expectation was based upon the classic model of neuronal trophic factors (reviewed in Barde, 1989). Based on the findings presented in this work, I was able to conclude that the innervation state of the target tissue has little or no obvious influence on the expression of cues used in target selection. While expressing a general, positive influence over growth, the HT is also able to constitutively express an HE-specific cue which slows growth, possibly aiding in the formation of mature synapses between the HE and the HT. It was also shown in this work, that the expression of a negative regulator of axon extension selective for the HE is not dependent on viable, living cells.
Previous studies on this project began the characterization of possible HT-
derived cues involved in HE development by using a co-culture system. From initial
experiments, it was determined that many adult axons (not only the HE) are
competent to extend onto HT, and that young, but not adult, HT is able to
support/stimulate outgrowth from adult axons. However, contrary to simple
predictions, the HE accounted for very little of the axonal outgrowth. The dye fills
showed that the HE is able to extend processes past the terminus of the ANR, but that
these extensions occur along the perineural sheath or as small caliber processes onto
the HT which are limited both in number and in length. The amount of HE outgrowth
was usually overshadowed by the exuberant outgrowth of other, unspecified axons
which do not normally grow onto the HT in vivo. The specificity with which the HE
(the motorneuron normally expected to contact the HT) did not sprout onto its target
was an extremely intriguing result which provided a powerful tool in the examination
of target signaling.

Before launching an extensive investigation into the inability of the HE to
elaborate extensively onto the HT in vitro, I performed a few simple, yet necessary
experiments to confirm and extend previous results suggesting that the basic result
was not merely an artifact of the experimental situation. I thought it remotely
possible that the lack of outgrowth might be attributed to the use of a fluorescent dye
in the prelabeling of the HE. This did not seem likely, however, due to the otherwise
healthy appearance of the cultured tissues. Visual inspection of the cultured tissues
revealed a normal morphology for the filled cells in that their axons and dendrites
were continuous and without the "blebby" appearance associated with dead or dying cells (Harik et al., 1999). Also, Hoechst staining of all nuclei disclosed the absence of pyknotic nuclei which might indicate unhealthy tissue. A set of experiments was also performed in which the cultures were generated without prelabeling HE neurons. Intracellular filling with fluorescent dextran following the normal incubation period reveal the same outgrowth pattern seen with prelabeling the neurons. Also, impalement of the cultured cells revealed normal electrophysiology, yet another indicator of the well being of the cultured tissue (Harik et al., 1999). In addition, several HEs were prelabeled with a nonfluorescent dye. When these preparations were examined, the level of outgrowth by the HE was again similar to what had been observed with the use of the fluorescent dye, whether as a pre- or postlabel (Crowley et al., 1999a; 1999b).

Another initial concern was that the culture situation may be lacking in some vital factor, one that is required for proper signaling by the HT or extension by neurites. To address this, I needed only to look at the results of other studies being conducted in the laboratory. In experiments examining HE outgrowth in situ, an adult ganglion was transplanted into embryonic animals in the vicinity of one of the host HTs. Even under these conditions, the HE was incapable of extensive outgrowth directed onto the HT despite being able to extend sprouts, indicating that lack of an essential nutrient in culture is not the reason for the poor outgrowth shown by the HE (Harik et al., 1999).
HE Specific Signal Is Not Regulated by Innervation

Elimination of these simple explanations as to why the HE shows very little outgrowth onto young heart tube in vitro left several intriguing possibilities. The experiments in this thesis were designed to test predictions from the two most seemingly parsimonious ones. The first of these was that the HE does not elaborate over the young heart tube because the heart tube has already been innervated. It may be that, prior to innervation, the heart tube is sending a signal of some sort which indicates the need for innervation i.e., a trophic signal (Dodd and Jessell, 1988; Goodman and Shatz, 1993). Once innervation has occurred, the signal may change to promote stable synapses and stop further extension along the heart tube. Anterograde signals are known to be sent from the growth cone to its target during synaptogenesis (Goodman and Shatz, 1993). During synapse formation, the axon terminal stimulates the initiation of a signaling cascade which leads to the development of pre- and postsynaptic specializations (Colman and Lichtman, 1993). Additionally, in vertebrate neuromuscular development innervation regulates the ability of muscle to present trophic factors (Thompson, 1985; Barde, 1989). Perhaps the HE requires a specific factor to stimulate growth, the expression of which is altered following innervation of the heart tube. It has been shown that the environment surrounding the developing neuron is continuously changing and thus, the growth cone and axon must change in response (Jessell, 1988; Dodd and Jessell, 1988).
To test this idea, I expanded upon the co-culture system of heart tube and ganglion explants developed in the laboratory (Harik et al., 1999). Young HT, deprived of local innervation, was cultured with adult ganglia containing prelabeled HEs. The denervation procedure, which involves removing large portions of the CNS prior to the emergence of the HT, is well established (Jellies et al., 1995). I hypothesized that the naïve HT would now be able to express the putative growth promoting cue and therefore stimulate lavish HE outgrowth onto the HT. However, the synaptically naïve HT failed to stimulate outgrowth that was anymore extensive than that seen previously using innervated HT. Generally speaking, the ability of the HE to extend onto tissue possessing either innervation state was limited to the formation of a few varicosities immediately upon exiting the ANR, or extension of 1-2 small caliber sprouts (Figure 6). Under both experimental conditions, there were examples of lavish outgrowth by the HE onto the target tissue. In most instances, however, this demonstration was accompanied by profuse outgrowth by unidentified neurons (Crowley et al., 1999a; 1999b). The modest outgrowth exhibited by the HE cannot be attributed to a physical barrier preventing exit from the ANR since the HE quite often sends elaborate processes back into or onto the perineuronal sheath. Also, several other neurons present in the ANR are able to extend lengthy processes out onto both the nerve sheath and the young HT.

While considerable variation in general outgrowth was seen in some groups of experiments, it could not be attributed to the experimental design. In other words, while some groups of experiments had significant outgrowth by the HE, it was
equivalent on both naïve and innervated tissue. I had thought it was possible that the naïve HT I was using was, in fact, receiving innervation extending from portions of the CNS left intact. However, this possibility was eliminated by other studies in the laboratory. In these investigations, animals which had received partial denervation prior to the emergence of the HT primordia were sacrificed following HT development. The animals were dissected and neuronal processes labeled with an antibody to acetylated tubulin. This experiment revealed no process elongation by the HE. The HE ordinarily innervates its segment of origin as well as half of one segment anterior and half of one segment posterior leaving a large portion of the HT uninnervated (Clark et al., 1999a; 1999b). HT used in co-culture experiments was taken from the center of the denervated region, not the ends. Consistent with these in vivo experiments, when I separately examined cultures done using HT from partial (6 segments) versus more complete (16 segments), there was no difference in HE outgrowth on naïve versus matched innervated HT.

Since similar outgrowth was seen on both innervated and naïve HT, two contrasting ideas concerning the influence of innervation on signaling by the HT are made very unlikely. The first is largely a formal possibility: that innervation does not up-regulate a positive growth regulator expressed by HT. Had this been the case, a greater amount of outgrowth onto the innervated as compared to naïve tissue would have been expected. The reverse is also true and was somewhat more surprising. Innervation does not down-regulate a positive growth factor either. If this were the case, outgrowth onto the naïve tissue would have been more exuberant than onto
innervated tissue. Thus, my original prediction has been falsified and it may be concluded that there is no compelling evidence for an innervation dependent influence over HE outgrowth onto the HT. This is in direct contrast to the current simple trophic model in vertebrate systems. In vertebrate models, the fate of the developing neuron at the time of target innervation is significantly influenced by the target muscle. It has been proposed that the nerve-driven activity of muscle fibers influences the production of a trophic agent which is required for the survival of the corresponding neuron (Thompson, 1985; Barde, 1989). Studies examining the effect of neurotransmission blockade showed the levels of neuronal cell death was decreased (Laing and Prestige, 1978; Pittman and Oppenheim, 1978), suggesting a role for electrical activity in the down regulation of a necessary trophic substance (Barde, 1989). Thus, inactive fibers express large amounts of the substance, while active fibers produce limited quantities (Thompson, 1985). The role played by target tissues in invertebrate models is not as well characterized. However, had this model held true for our design, a difference in outgrowth patterns between innervated and naïve HT would have been seen, with the expectation that naïve tissue would be better able to stimulate outgrowth by the HE than would the previously innervated tissue.

The results achieved in these investigations are consistent with the hypothesis elaborated previously (Harik et al., 1999) and actually model quite accurately what occurs during neurodevelopment in vivo. We suggest that the HT expresses a contact dependent, growth permissive cue(s) that stimulates and/or supports axonal sprouting from a wide variety of neuronal types. At the same time, we suggest that the HT
could be constitutively expressing a negative regulator of rapid growth which acts on
the HE. While many neurons appear able to elaborate onto the HT in vitro, the
hypothesis demands that in vivo the HE is the only neuron with available, competent
processes in the vicinity of the emerging HT (Harik et al., 1999). Perhaps the HE
responds initially to the general growth cues presented by the HT but upon making
contact, responds to the putative HE-specific cues which slow growth by the HE and
perhaps promotes the formation of stable synapses or more branching. Such a
scenario has previously been suggested based on studies done in embryonic chicken
(Lance-Jones and Landmesser, 1981; Tosney and Landmesser, 1985). Likewise,
“stop-signals” promoting synaptogenesis have been proposed for vertebrate CNS and
neuromuscular development (Mason et al., 1997). Results of my investigations lend
support to the idea that pathways exist which appear to provide a nonspecific,
permissive substrate for general axonal outgrowth while targets also contain specific
cues which act on a subset of growth cones allowing for slowed growth and
stabilization of growth cone filopodia. This specific interaction between the
developing neuron and its environment has been further described as utilizing both
anterograde and retrograde communication; examples of such interchange exist
within several experimental models (Frank and Wenner, 1993; Goodman and Shatz,
1993). Studies examining nerve-muscle interactions at the developing vertebrate
neuromuscular junction have shown that cell-cell communication is crucial to the
development and maintenance of synapses (Hall and Sanes, 1993). Fernandes and
Keshishian (1998) showed that myoblast pool size and initial fiber formation depend
on the presence of the nerve and that, in turn, secondary branching and synapse formation by the nerve depended upon the developmental stage of the muscle. The authors therefore concluded that surface molecules on the target muscle play a role in the establishment of nerve processes. The B19 motoneuron of Heliosoma interacts with muscle other than its eventual target, however, it selectively forms appropriate connections with its definitive target muscle. (Zoran et al., 1990; Zoran and Poyer, 1996) Furthermore, it has been shown that retrograde communication between the B19 neuron and target muscle is important for synapse formation in this system (Haydon and Zoran, 1994). Additional examples of retrograde communication between target tissue and developing neurons are found in both the Rz (Glover and Mason, 1986; Jellies et al., 1987; Loer et al., 1987; Macagno et al., 1986; French et al., 1992) and RPE neurons (Baptista and Macagno, 1988) both of which innervate the sex organs of the medicinal leech.

Formation of HE-HT Synapses May Be Mediated by Matrix Molecules

Previous studies have implicated leech extracellular matrix (ECM) as having profound influences on the amount and pattern of axon outgrowth (Letourneau et al., 1992; F. de-Miguel and Vargas, 1997; F. de-Miguel, 1997). Therefore, in a set of preliminary studies to characterize the target influences, I repeated the cultures using HT that had been killed by freezing and thawing. In these experiments, embryonic HT was subjected to repeated freeze/thaw cycles which served to kill the tissue. The dead HT is therefore incapable of producing any new signal, yet some membrane
bound proteins and ECM, which may be involved in signaling, are left intact (Merz
and Drapeau, 1994).

Co-cultures using dead HT again showed only modest outgrowth by the HE
onto the target tissue. In the majority of preparations, extension by the HE onto the
HT was limited to formation of varicosities immediately at the cut end of the ANR or
1-2 short sprouts onto the HT (Figure 9).

The inability of the HE to extend elaborate processes onto the dead HT is
quite interesting. This result confirms that the HT is utilizing a contact-mediated
mechanism to support outgrowth of the HE. It also strongly suggests that active
secretion of factors from living muscle is not necessary for these initial stages of
target selection. Similar studies examining the formation of the Rz-P synapse in the
medicinal leech found that glycoproteins on the Rz cell surface mediate cell
recognition during formation of the Rz-P synapse (Merz and Drapeau, 1994). Based
on investigations in Drosophila embryos, Johansen et al. (1989) suggest that the most
elementary conditions of mature neuromuscular innervation are formed due to
recognition by the motorneuron growth cone of the surface features of the muscle
membrane. Letourneau et al. (1994) has studied extensively the ability of the growth
cone to bind to ligands present on cell surfaces and to extracellular matrix (ECM)
components such as fibronectin and laminin. Bonds formed with these molecules are
capable of stabilizing the filopodia of the developing neuron. Taken together with
this information, our results implicate the use of a contact-mediated signaling event
that may precipitate the formation of HE-HT synapses. Furthermore, the signal
survives in the matrix despite the loss of intact, viable cells. Precedent exists for such speculation. Studies examining the location of factors involved in the regeneration of neurons found that regenerating neurons contact the basal lamina at the original synaptic sites, indicating that factors directing axon extension are not only present at synaptic sites, but are also maintained externally on the myofiber. Furthermore, the regenerating neurons are able to form nerve terminals even though their postsynaptic muscle target is absent. Such findings support the suggestion that the integrity of the postsynaptic cell is not essential for directing the reinnervation of target tissue (Marshall et al., 1977; Sanes et al., 1978).

Do Embryonic Neurons Elaborate More Profusely Than Adult In Vitro?

A second explanation for the deficiency in HE outgrowth lies in the neuron itself. It may be that as the neuron ages and innervates tissue, it loses the ability to respond to signals produced by the embryonic heart tube. Perhaps some retrograde signal following synapse formation alters the ability of the mature neuron to grow onto the explanted HT, or CNS connections irreversibly induce changes in the motor neuron. In other words, the adult neurons used in my study were not naive either. To investigate this, I again used a co-culture system. For these experiments however, embryonic HE cells were prelabeled with Texas Red-dextran prior to the emergence of the HT primordia and chains of these ganglia cultured with normal, innervated heart tube from a later staged embryonic animal.
While this experiment was designed to test the prediction that naïve HE neurons will extend profusely on embryonic heart, the co-culture situation, while intriguing, proved inadequate. The attempted experiments had minimal contamination, the ectopic tissues were able to stick to the polylysine coated coverslips, the acetylated tubulin antibody was working, and Hoechst staining of nuclei revealed viable neurons and muscle cells. Although the preparations initially appeared comparable to those using adult CNS, closer inspection revealed discontinuous “blebby” axons as if they were unable to continue growing after removal from the animal. This negative response by embryonic neurons to the culture conditions was examined more closely to assure myself that the result was independent of technique. To determine whether holding tissue in refrigeration prior to plating was causing the effect, parallel cultures were performed in which embryonic CNS was removed from host and plated immediately in culture filled dishes; no difference in neuronal response was seen. Additionally, both Texas Red injection and FITC secondary label were used to tag neurons resulting in similar results. Finally, the presence or absence of target tissue in the cultures did not effect the health of the embryonic neurons.

Experiments performed in situ show that segments of embryonic nerve cord can be rescued, but this may only be when it becomes vascularized by the host animal (Harik et al., 1999; Jellies et al., 1999). This result is intriguing as it suggests that embryonic neurons may depend upon as yet unidentified trophic factors. General
growth cues may be present in the blood of the animal that are not provided in culture.

The issue of embryonic neuronal response to embryonic HT can be addressed by experiments performed \textit{in vivo}. As mentioned previously, other experiments in the laboratory have shown that the amount of HE extension along the HT in the developing embryo is limited to its segment of origin plus one-half segment anterior and one-half segment posterior. Also, the length of the HE extension is already established in the body wall prior to the emergence of the HT primordium (Clark \textit{et al.}, 1999a; 1999b). Once the HT emerges, the HE processes are simply redirected from the body wall onto the developing HT and additional growth along the HT does not take place. Thus, while I was unable to employ embryonic CNS \textit{in vitro}, the \textit{in vivo} manipulation eliminates the probability that embryonic HEs respond to HT differently than adult.

\textbf{Future Studies}

Although an extensive characterization of the contacts the HE forms onto the HT is beyond the scope of this project, several methods could be employed in future studies to test the hypothesis regarding the presence of a target-derived cue which is able to stimulate the formation of stable HE synapses \textit{in vitro}. Transmission electron microscopy could be used to determine whether the pre- and postsynaptic substrates of stable synapses are present early. By examining these contact points at the electron
microscopic level, it would be possible, for example, to detect the presence of synaptic vesicles in the end plate region of the HE motorneuron.

Electrophysiology is another possible means of determining synapse formation. In utilizing this method, both the presynaptic motorneuron and the postsynaptic muscle fiber would be impaled with glass microelectrodes. Current would then be passed into the HE. If this stimulation was able to cause a potential change in the postsynaptic muscle fiber it could be concluded that the HE is able to form stable electrical synapses with the HT \emph{in vitro}.

One could also perform Dil injections of the cultured HT. As described previously, Dil is a lipophilic dye which, when injected into the HT lumen, is transported across cells with closely apposed membranes (the synapses of neuromuscular junctions) therefore neurons forming membrane appositions should be labeled. It is suspected that the elaborate general outgrowth seen on cultured HT is simply due to a permissive substrate being provided for neurons to grow upon and their lengthy processes are the result of the inability to form functional synapses with the muscle tissue. Along the same lines, the inability of the HE to extend elaborate processes is the suspected result of immediate initiation of synapse formation.

Additional studies do need to be performed to address the uncertainties concerning synapse formation by the HE, as well as other neurons exhibiting outgrowth, onto the HT \emph{in vitro}, and also to further characterize the signaling mechanisms employed in target selection. The results presented here, however, support the hypothesis that the HE, along with many other neurons, is able to respond
to general growth promoting cues provided by the HT; however, unlike the other neurons, the HE is also able to respond to a constituitively expressed negative regulator of growth.
Appendix A

The Use of HRP for Double Labeling Axonal Outgrowth
Using HRP as a label to identify outgrowth by both the HE and unidentified neurons was attempted in an effort to simultaneously quantify HE outgrowth and confirm the health of the cultured tissue. Since fluorescent labels tend to fade upon exposure to the UV light, quantifying outgrowth when such tags are used is impractical. HRP, being a much more resilient label, is preferred for such analysis; however, simply labeling the HE processes leaves concerns about the overall condition of the preparation. I thought that if general outgrowth could also be labeled by a more stable staining process, I could assure myself that the tissues in culture were healthy and that the results obtained were not due to some undetected weakness in tissue integrity. To this end, a processing method was used which, in theory, would label HE processes black and general processes brown.

Materials and Methods

Biotin-conjugated dextran was used to fill HE neurons and these ganglia used for co-cultures following the method described previously. Fixed tissues were subsequently incubated with HRP-streptavidin (Vector) 1:500 in PBS with 1% Triton X-100 and 0.001% sodium azide overnight in the refrigerator. The preparations were rinsed repeatedly with PBS followed by Tris. The tissues were then incubated with DAB (.5 mg/ml Tris; Gibco) for 15 minutes at 4°C after which, 2% CoCl₂ was applied for approximately 2 minutes. Reaction with H₂O₂ was stopped with cold Tris once a black precipitate was visible. The preparations were then rinsed extensively in Tris followed with PBS. The monoclonal antibody ACT was diluted 1:1000 in PBS
with 10% goat serum, 1% Triton X-100, and 0.001% sodium azide. Incubations were carried out overnight at 4°C. Tissues were extensively rinsed with PBS prior to the addition of the secondary antibody. HRP-conjugated (at 1:500; Molecular Probes) goat anti-mouse IgG was diluted in the same buffer described above and again allowed to incubate overnight at 4°C. Preparations were rinsed extensively with PBS followed by Tris. The tissues were again incubated with DAB (.5 mg/ml Tris; Gibco) for 15 minutes at 4°C. Reaction with H₂O₂ was stopped with cold Tris once a brown precipitate was visible. The preparations were then rinsed extensively in Tris, dehydrated in ethanol, cleared in methyl salicylate, and mounted in Cytoseal 60.

Results

While a few of the preparations turned out well enough to distinguish between the two labels, most did not. In the majority of the preparations utilizing this method, I could not comfortably determine which processes were those of the HE and those representing general outgrowth (Figure 13). This was especially difficult in those examples showing elaborate extension onto the HT. While this is a method commonly used and with much success, in this particular experimental design, it did not prove adequate to fulfill our needs. There was as much subjectivity in deciding whether label was dark, as in our simpler qualitative assessment.
Figure 13. HRP Double Label Cannot Distinguish the HE Motorneuron. HEs were injected with biotin-dextran, labeled with HRP and reacted with DAB and cobalt chloride for a black label. General outgrowth was subsequently visualized using ACT followed by an HRP linked secondary antibody and reacted with DAB for a brown label. In most preparations, the two labels were indistinguishable from one another.
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