Stage-Dependent Expression of Target-Derived Influences Stimulates Axon Outgrowth in *Hirudo Medicinalis*

Tina M. Harik  
*Western Michigan University*

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STAGE-DEPENDENT EXPRESSION OF TARGET-DERIVED INFLUENCES STIMULATES AXON OUTGROWTH IN *HIRUDO MEDICINALIS*

by

Tina M. Harik

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Tina M. Harik
STAGE-DEPENDENT EXPRESSION OF TARGET-DERIVED INFLUENCES STIMULATES AXON OUTGROWTH IN Hirudo medicinalis

Tina M. Harik, M.S.
Western Michigan University, 1998

Our lab has been investigating the development of neuronal phenotype and circuitry in Hirudo medicinalis. Early in embryogenesis the heart excitor (HE) motor neuron selectively extends multiple arbors into the body wall prior to heart tube (HT) formation. HE contact with HT primordium at later stages of development results in remodeling such that arbors are elaborated on the HT, while those in the body wall are retracted. HT-derived cues are necessary for HE remodeling (Jellies, 1995. Am. Zool. 35:529; Jellies and Kopp, 1995. Invert. Neurosci. 1:145). HT and body wall explants were co-cultured to investigate the possible influences of target-derived cues in altering axon growth. Axon outgrowth was examined by labeling with a monoclonal antibody (mAb) directed against acetylated tubulin, and nucleated cells were labeled with bis­benzimide. Initially, adult tissues were co-cultured and most showed minimal axonal outgrowth, even in cases where target muscle was in close proximity. These adult axons, however, sprouted and grew upon the perineural sheath. In contrast, co-culturing adult ganglia with embryonic or juvenile HT and body wall resulted in profuse axonal outgrowth on the younger target as well as extensive growth on the perineural sheath. The identities of the axons elaborating on the young HT or body wall have not yet been determined, but these results provide strong direct evidence that target-derived factors can effectively stimulate axon outgrowth in vitro. Also, they argue that the adult neurons remain competent to respond to HT-derived factors, but that HT may lose its ability to express or present these factors.
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How a developing neuron interprets the information from its intrinsic properties and its environment to produce a correct circuit is a fundamental question of developmental biology. There are at least three major steps involved in the development of the specificity of synaptic connections: (1) pathway selection, (2) target selection, and (3) address selection (Goodman and Shatz, 1993). Previous studies suggest each step may be executed by one or a combination of three possible mechanisms (Lance-Jones and Landmesser, 1981; Jellies and Kristan, 1988; Jessell, 1988; Dodd and Jessel, 1988; Lander, 1990; Frank and Wenner, 1993; Goodman and Shatz, 1993; Johansen et al., 1994; Jellies and Johansen, 1995; Jellies, 1995; Huang et al., 1997; Kafitz and Greer, 1997).

During pathway selection, growth cones must grow long distances through different environments to find their targets. Within these environments the growth cone must correctly navigate or be capable of later correcting errors in projection. Growth cones respond in unique ways to various cues in their surroundings that often result in stereotyped pathways and patterns of synaptic connectivity. These cues may be local or long distance, contact mediated or diffusible, including: guidepost cells, chemical cues, and structures or scaffolds such as pioneering neurons (Lance-Jones and Landmesser, 1981; Jellies and Kristan, 1988; Jessell, 1988; Dodd and Jessel, 1988; Lander, 1990; Frank and Wenner, 1993; Goodman and Shatz, 1993; Johansen et al., 1994; Jellies and Johansen, 1995; Jellies, 1995; Huang et al., 1997; Kafitz and Greer, 1997).
One of the possible mechanisms of pathfinding involves contact mediated cues and selective adhesion of filopodia as an explanation of directed outgrowth of processes towards their target. In leeches the major projections of peripheral sensory neurons divide into four tracts differentiated by surface expressed antigens (Zipser and McKay, 1981; Johansen et al., 1985; Johansen et al., 1992). Johansen et al., (1994), have shown these antigens are necessary for peripheral sensory neuron axon navigation to the CNS. Monoclonal antibodies were used to bind the surface antigens and prevent their interaction with other molecules. The sensory axons were found to extend upon aberrant pathways. These results suggest these surface bound molecules provide cues to establish the directed growth of peripheral sensory neurons along their proper pathway to their CNS target (Johansen et al., 1994; Jellies and Johansen, 1995).

Another example of this are netrins. Netrins from a variety of organisms are secreted molecules that can attract or repel growth cones. Lauderdale et al., (1997), have shown netrins are expressed in dynamic patterns in the zebrafish embryo and suggest that they may act to delineate specific pathways and stimulate axonal outgrowth. \textit{In vitro} studies of Kafitz and Greer, (1997), suggest that laminin provides a favorable substrate for the growth and direction of extension of the primary neurite from rat olfactory receptor cells.

Target-derived cues may also have an influence on pathfinding. Each leech ganglion possesses two Retzius neurons that, in most segments, innervate body wall muscle in their own segments and two adjacent segments. However, in segments 5 and 6 these neurons navigate from the CNS to innervate the reproductive organs at the midline (Jellies and Kristan, 1988; Loer and Kristan, 1989; Jellies, 1995). Transplantation and ablation studies in developing leech embryos show that the Retzius neuron is influenced by contact with their peripheral targets. When sexual organs were ablated prior to contact with the Retzius cells, these cells revert to innervating body
wall. Also, when the sexual organs were transplanted to other segments in the leech, the Retzius neurons in those segments extended to innervate the sex organs, not body wall.

Landmarks such as guidepost cells and tissue structures have been found to play a role in axon pathfinding in many animals. In situ experiments in grasshopper limb buds show that guidepost cells and segment boundaries play an important role in the development of the Ti1 neuron in that they correctly orient the Ti1 growth migration through the limb bud to its correct target (O'Connor et al., 1990). Greenspoon et al., (1995), demonstrated notochord and floorplate cells in the zebrafish embryo participate in guiding specific growth cones at the ventral midline of the spinal cord.

Target selection is the step in which the growth cone contacts a target, and initial synapses form and become stable. These patterns of connection may later undergo a remodeling called address selection in which certain terminals expand and others retract further sharpening the selection of one of several possible targets. Permissive and general pathways may be utilized by many growth cones, but only a subset may follow specific cues to reach their proper targets. Evidence supports several possible mechanisms for target selection (Lance-Jones and Landmesser, 1981; Jellies and Kristan, 1988; Jellies and Kristan, 1988; Dod and Jessel, 1988; Lander, 1990; Frank and Wenner, 1993; Goodman and Shatz, 1993; Johansen et al., 1994; Jellies and Johansen, 1995; Jellies, 1995; Huang et al., 1997; Kafitz and Greer, 1997). Studies of the innervation of the leech sex organs provide some of this evidence. As mentioned before Retzius neurons normally innervate body wall. However, in segments 5 and 6 once Retzius growth cones contact the sex organs a target-derived retrograde influence results in a remodeling of the axonal processes. These neurons undergo a morphological change and retract terminals in the body wall and extend terminals upon
the male and female sex organs in segments 5 and 6 (Jellies and Kristan, 1988; Loer and Kristan, 1989; Frank and Wenner, 1993; Jellies, 1995). It has also been found in snails (Heliosoma) that retrograde target-derived signals transform growth cones into functional synapses with its muscle target (Zoran et al., 1990). For example, the buccal motoneuron 19 requires contact with appropriate target muscle before its presynaptic terminals acquire the ability to release neurotransmitters (Zoran et al., 1990).

Address selection occurs as axonal terminals retract and expand to select a specific subset of cells from within the overall target. This refinement often relies upon competition with surrounding inputs (Goodman and Shatz, 1993). Drosophila embryonic growth cones initially sample many potential muscle targets before forming stereotypic synapses with one or a few muscle fibers (Davis et al., 1997). Individual muscle fibers have distinct and anatomically stereotyped neuromuscular junctions which are the direct consequence of growth cone behavior (Johansen et al., 1989a; Johansen et al., 1989b; Halpern et al., 1991). It has been shown Fasciclin II (FasII) expression is required presynaptically and postsynaptically for synaptic stabilization and has a profound effect on the pattern of synapse formation (Davis et al., 1997). Fas II is normally expressed in low levels on the muscle before synapse formation. During synapse formation Fas II concentrates at the synapses and disappears from the rest of the muscle fiber. It was also shown that differential expression of Fas II on neighboring muscle fibers results in dramatic shifts in address selection. Davis and Murphey, (1994), also suggest that retrograde signaling is required to form and maintain functional synapses in a range of invertebrates and vertebrates.

Address selection is also extremely important in the development of vertebrate visual systems. Thousands to hundreds of thousands (depending on the species) of retinal ganglion cells send their axons the central visual structures where the final
accuracy of their connections determine the animals ability to see and resolve fine
details of the visual world. Initially the axons arborize over a larger area of the central
visual structure that results in a coarse topographical map, thus coarse vision. As the
visual system develops and arborizations are remodeled to specific areas a fine-grain
retinotectal map gradually results in higher resolution vision than before address
selection (Goodman and Shatz, 1993).

Axon guidance molecules and molecular substrates for target selection and
address selection are not unique to the nervous system. These molecules are found in
many other tissues and are involved in cell-cell interaction, vesicle transport, cell
membrane dynamics and other physiological phenomena. Mechanisms by which axons
are guided may also apply to other systems, thus, interests in stereotyped pathfinding,
patterns of neuronal connectivity, and target selection are foci of much research
involving motility in general.

This project will characterize neuronal outgrowth in Hirudo medicinalis in an in
vitro system as a part of a long term goal of identifying the molecular substrates of
pathfinding and target selection. The specific experiments are driven by the hypothesis
that HE development is influenced by HT-derived cues, but the study examines general
issues of axonal outgrowth. Specifically, my experiments will examine the stage-
dependent expression of heart tube-derived influences stimulating axon outgrowth in
the leech H. medicinalis.

Animal Model and Experimental Background

Hirudo medicinalis is an excellent model system in which to conduct this study.
Neurons are relatively large (10µm to 60µm diameter) but relatively few in number
(400 in each ganglion) which facilitates identification. It is possible to identify, by
simple visual inspection, individual cells reliably in the leech ganglia according to their shapes, sizes and soma positions (Muller et al., 1981) (Figure 1). Electrical recordings from the individual neurons can confirm the identification of the neurons (Muller et al., 1981). Many individual neurons are identified and well characterized, such as the heart excitor (HE), Retzius and annulus erector (AE) neurons as well as many touch, pressure and nociceptive cells. Also, heart explants, CNS explants, and individual neurons can be manipulated *in vitro* and survive well (Chiquet et al., 1988; F. de Miguel and Vargas, 1997; Fernandez-de-Miguel, 1997; Harik and Jellies, 1997a; Harik and Jellies, 1997b; Jellies et al., 1996a).

The central nervous system of the leech consists of a chain of 32 ganglia that extend along the ventral midline of the animal, as well as head and tail brains consisting of condensed ganglia (Figure 2). Each ganglion in midbody segments 3-18 (M3-M18) has a bilaterally paired heart excitor (HE) motor neuron (Figure 1). The HE motor neurons drive coordinated rhythmic contractions of the heart tubes, which are bilaterally paired contractile vessels that extend the length of the animal (Figure 2). The heart tubes are thus under the influence of a central pattern generator (CPG) (Thompson and Stent, 1976; Stent et al., 1979) which drives the HE's. HE's are also electrically coupled to each other within the CNS in their own segments (Tolbert and Calabrese, 1985).

By combining intracellular dye-filling and antibody staining of developing embryos it has been possible to examine the sequence of growth cone extension and target selection by the HE (Jellies et al., 1996b; Jellies and Kopp, 1995). This stereotyped circuitry arises as the HE begins to project an axon out the already pioneered anterior nerve root (ANR) at approximately embryonic day eight (E8) (Jellies and Kopp, 1995; Jellies et al., 1995; Jellies et al., 1996a; Jellies et al., 1996b; Jellies et al., 1996c) (Figure 3). The HE growth cones elaborate extensively, branching off the established
Figure 1. HE's Can Be Identified by Axon Pathway and Location in Ganglion With Respect to Other Neurons. Diagram of the leech mid-body ganglion. (A) Dashed lines represent the packet boundaries within the ganglion. A single glial cell envelopes each packet, and each ganglion has six packets. The HE is located in the antero-lateral packet. Light gray circles represent other large neurons in the ganglion. ANR is the anterior nerve root, PNR is the posterior nerve root. (B) This diagram illustrates the pathway of the bilaterally paired HE axon within the ganglion. The HE axon exits the ganglion ipsilaterally through the ANR. It is the only axon in the ganglion to follow this pathway, therefore the HE and its axon can be readily identified.
Heart Tubes Are Part of a Closed Circulatory System Under Direct Neural Control. This diagram illustrates the relationship of heart tubes to the central nervous system. Ganglia of the ventral nerve chord (circles) are contained in a blood sinus along the ventral midline. There is also a conducting sinus along the dorsal midline. The bilaterally paired heart tubes are found in mid-body segments 3-18 (shaded areas) and connect with the conducting sinuses at the head and the tail.
Developmental Progression of Changes in HE Motor Neurons as a Result of Contact With the HT. Starting E8 (1) The HE sends out an axon. There is an initial phase of extensive stereotyped branching (2,3). Early on E12 (4) the HT's emerge. HE contact with emerging HT results in a rapid remodeling of peripheral arbors (5,6). At about E16 the HE makes connections with the CPG and undergoes electrical coupling to the contralateral HE (6,7).

pathways to pioneer longitudinal stereotyped tracts, including the correct tract in the vicinity of where the heart tube will arise. These supernumerary arborizations are in stereotyped locations, but not related to the HT, and are always parallel to the long axis in the body wall muscle. This suggests the arborizations are not random projections but instead are the result of discrete tract formation during embryogenesis (Jellies et al., 1996b; Jellies et al., 1996b). During E12 the heart tube primordium begins to emerge just superficial to the longitudinal body wall muscle between the mesoderm of the body
and the endoderm of the gut. Between E12 and E14 there is an extensive elaboration of HE terminals over the surface of the emerging heart. Simultaneously, the HE undergoes morphological changes which results in the retraction of axons in the supernumerary tracts while increasing the extent of arborizations on the HT. At about E16 there is electrophysiological evidence of non-rhythmic inhibitory input to the HE. The input becomes increasingly more rhythmic between E16 and E18, resembling the mature circuit by E23, indicating connections have been made with the CPG (Jellies et al., 1992). Because this anatomic remodeling occurs before the centrally driven pattern of rhythmic activity, it is suggested that specific target interactions may play a role in the anatomical patterning and the development of central connectivity.

Embryonic HE motor neurons were surgically deprived of heart target to examine the role of possible heart-derived cues on HE development (Jellies and Kopp, 1995). In these animals the HE shows development similar to that of embryonic stages in that the supernumerary projections are not retracted, and the HE’s show tonic excitability as though not being regulated (or synaptically driven) by the CPG. Also a network of novel CNS projections is observed (Jellies and Kopp, 1995). These results support a role for a strong retrograde influence of target-derived cues on the HE development. There remain a number of important issues to address. Among them are whether the heart-derived influence is a result of direct contact or a response to a diffusible factor. Additionally, neither the temporal expression nor the identity of HT-derived factors has been established.

For this study whole ganglion explants were used as a model in a first step in examining the role of HT-derived factors on general axonal outgrowth. The developed in vitro system allows the HT and CNS to be isolated in order to begin to address general characteristics of molecular substrates of pathfinding and target selection. Also, co-cultures of different staged-target tissue facilitates examining the stage-dependent
expression of target-derived cues and their influence over axonal outgrowth. Future studies are planned to study the influences of these cues specifically on the HE motoneuron.
CHAPTER II

MATERIALS AND METHODS

Animals

Embryos and juveniles of the medicinal leech, *Hirudo medicinalis*, were obtained from a breeding colony at Western Michigan University as previously described (Jellies et al., 1987), maintained at room temperature and staged in days of development (Fernandez and Stent, 1982). Formation of the head sucker indicates approximately day 16 of embryogenesis (E16). A seam extending down the dorsal midline where the two edges of body wall fuse indicates approximately E20 of embryogenesis. The termination of embryogenesis is E30. Embryos used in these studies were E16-E20. Juveniles were 30-60 days post-embryonic. Adult animals were obtained from the WMU breeding colony and Leeches USA (Westbury, N.Y.)

Before dissection, adult and juvenile animals were anesthetized in cold, sterile normal *Hirudo* ringer (in mM, 115 NaCl, 4 KCl, 1.8 CaCl$_2$·H$_2$O, 1.5 MgCl$_2$·6H$_2$O, 10 glucose, 4.6 Tris maleate, 5.4 Tris base) for 10-20 minutes. Embryonic animals were anesthetized in cold 8% ethanol/92% saline.

Dil Injections

A dorsal incision was made from the head to the tail sucker along the mid-line. Heart tubes were exposed and the epithelial gut lining was removed. Dil (Molecular Probes) stock at 10 mg/ml in 100 (µl) DMSO:ethanol (1:1) was diluted in phosphate buffered saline (PBS) (.1M) 1:10 and pressure injected into the heart tubes of dissected
leeches (Jellies, 1995). Preparations were fixed in cold 4% paraformaldehyde overnight then rinsed well in PBS and incubated at 37°C for 2 to 7 days. Preparations were mounted in 75% glycerol/25%PBS on glass slides (Fisher) and visualized using a Nikon epifluorescent microscope through the rhodamine filter.

Tissue Explants

Tissues were plated on poly-L-lysine (Sigma) coated glass coverslips (Assistant Inc.) in sterile plastic culture dishes. Coverslips were autoclaved, and poly-L-lysine solution (2mg/ml water) was sterile filtered (Sterile Acrodisc, Gelman Sciences) directly onto the cover slips and put into the refrigerator for 1-24 hr. The coverslips were rinsed extensively in water (Fisher, HPLC grade) and stored in the refrigerator for up to two weeks before use.

Coated glass coverslips were placed in sterile plastic culture dishes. Approximately 2 ml culture medium was then sterile filtered (Sterile Acrodisc, Gelman Sciences) directly into the dish. Culture medium consisted of Leibovitz L-15 medium (Sigma) supplemented with 6mg glucose/ml (Sigma) and 6 µg glutamine/ml (Sigma), diluted with sterile saline (3:1). Penicillin-streptomycin (Sigma) was added to obtain a concentration of 90% culture solution and 10% pen-strep for the final culture medium.

Seven different experimental culture situations were used in this study: (1) adult ganglia alone, (2) co-culture of adult ganglia and adult HT, (3) co-culture of adult ganglia and juvenile HT, (4) co-culture of adult ganglia and embryonic HT, (5) co-culture of adult ganglia and adult body wall, (6) co-culture of adult ganglia and juvenile body wall, and (7) co-culture of adult ganglia and embryonic body wall.

Animals were dissected as described earlier. The epithelial gut lining was removed as well as the protective blood sinus of the CNS. Explants were removed
with microdissection tools (sterilized in 95% ethanol and rinsed in sterile Ringer Solution). The connectives of the ganglia were cut just anterior and posterior to the ganglion, the anterior nerve roots were cut just proximal to the first bifurcation, and the posterior nerve roots just distal to the first bifurcation (Figure 4). The explants were plated on poly -L-lysine coated glass coverslips ventral side down in culture medium. Nerve roots were arranged with glass microneedles to extend along the substrate in close proximity (0µm to 200µm) to the HT. After 24 hours in culture 200 µl goat serum (Sigma) and 200µl ITS liquid media supplement (Sigma) were sterile filtered into each culture.

Cultures were maintained for 5 to 10 days at room temperature then processed for immunocytochemistry. Tissue adhesion was very delicate, therefore it was necessary to keep cultures on anti-vibration tables (Newport) to minimize movement that may have disturbed the explants.

Immunocytochemistry

Cultures were fixed overnight in 4% paraformaldehyde in 0.1 M phosphate buffer (pH7.4) in the cold. Preparations were then rinsed extensively with PBS before further processing.

Cultures were incubated in bis-benzimide (Sigma) .01mg/ml Tris for 15 minutes at room temperature to label nuclei. They were rinsed extensively in Tris before processing with antibodies.

A monoclonal antibody (mAb) directed against acetylated tubulin (ACT, Sigma) was used to label central neurons and their axonal projections (Jellies et al., 1996b). The primary Ab was used at a concentration of 1:1000, in 10% goat serum, 10% Triton X-100, .001% sodium azide, in PBS. Incubations were carried out for six hours to
Figure 4. Tissue Culture Process. A) This diagram illustrates two mid-body segments of the leech. The body was cut down the dorsal mid-line and pinned ventral side down. Only the CNS and heart tubes are shown for simplicity. The dotted lines indicate where the ganglia were cut, solid lines indicates where the HT explants were removed, and the dotted diamond indicates where the body wall explants were removed. B) These diagrams illustrate the composition of the culture situations. Ganglia were cultured alone, ganglia were co-cultured with different aged heart, and adult ganglia were co-cultured with different aged body wall. Explants were plated on poly-L-lysine coated coverslips and arranged with micropipettes in culture dishes.

overnight in the refrigerator. Preparations were again rinsed extensively in PBS before addition of the secondary antibody. Texas Red-conjugated goat anti-mouse IgG (Molecular Probes, Sigma) was used at a concentration of 1:500, in 10% goat serum, 10% Triton X-100, .001% sodium azide, in 80% PBS. Preparations were again rinsed
extensively in PBS before mounting in glycerol. Mounted preparations were visualized using an epifluorescent Nikon microscope with appropriate filter sets.
CHAPTER III

RESULTS

Dil Injections

Dil injections were performed on adult animals in order to confirm the specific innervation of the HE on the HT in vivo. Dil is a lipophilic dye, which is transported from the HT lumen through the synapses of neuromuscular junctions (Jellies, 1995). Therefore, the Dil should label neurons which directly form membrane appositions such as electrical synapses on the HT. In previous studies intracellular dye-filling and electrophysiology of the HE suggested the HE specifically innervates the HT (Thompson and Stent, 1976; Maranto and Calabrese, 1984). Dil injections in the HT were observed only to retrogradely label the HE motor neuron.

Due to the importance of the hypothesis that the HE was the only neuron to innervate the HT I extended previous Dil experiments (Jellies, 1995) in order to confirm this specificity. In all instances where the injected Dil was limited to the heart either no neurons were labeled or only a single HE axon and HE soma (n=13) were labeled, and the HE terminals were limited only to the HT (n=13) (Figure 5). This would suggest the HT is the ultimate and sole proper target for the HE motor neuron. Also, no other neurons were retrogradely labeled indicating the HE is the only neuron to innervate the HT. My results confirm previous work on this system and support the hypothesis that the HE innervation of the HT is specific and the HT is not directly innervated by any other neurons.
Figure 5. A Technique Using Dil Injections Into the Heart Lumen Retrogradely Labels HE's. (top figure) In all instances where the injected Dil was limited to the heart either no neurons were labeled or only the HE was labeled and HE terminals were limited only to the HT. (bottom figure) Diagram showing Dil injection in HT and HE retrograde label.
Tissue Culture

To test the hypothesis that HT-derived cues stimulate axon outgrowth in vitro I expanded upon a co-culture system (Chiquet and Acklin, 1986; Chiquet et al., 1988; Masuda-Nakagawa et al., 1990; Acklin and Nicholls, 1990; Fernandez-de-Miguel, 1997; F. de Miguel and Vargas, 1997) using HT or body wall and ganglion explants. The different aged explants allowed stage dependent expression of HT-derived influences over the axon outgrowth to be examined in vitro.

By using ACT antibodies I examined general axon outgrowth in vitro. Bis­benzimide, specific for nucleic acids, was used to stain the nuclei of the cells of the explants, especially the HT. By using ACT antibodies in conjunction with bis­benzimide, I could visualize the axonal outgrowth on the HT.

Adult Ganglion Cultures

Adult ganglia were cultured alone to determine the capability of axons to grow without target-derived cues in the culture. Most ganglia (20/28) showed some, but minimal axonal outgrowth (Figure 6). Even in cases where adult HT was co-cultured in close proximity (less than 10 µm) with the nerve (13/28), little outgrowth was observed from the nerve on the HT surface (Figure 7). Of the 13 preparations with outgrowth, 7 showed no axonal outgrowth on the HT, while in 5/13 there was extension of 1-2 short sprouts. These adult axons, however, sprouted and had limited growth on the perineural sheath of the nerve. Thus, they survived well and retained their ability to extend (Harik and Jellies, 1997a; Harik and Jellies, 1997b).
Figure 6. Adult Ganglia Cultured Alone Exhibit Limited Outgrowth. Nerves were labeled with anti-acetylated tubulin (red), arrow indicates cut nerve ends. When ganglia were cultured without heart, axonal outgrowth was limited. This image shows limited perineural outgrowth in absence of heart.

Figure 7. Adult Ganglia in Co-culture With Adult HT Exhibit Limited Outgrowth. Nerves were labeled with anti-acetylated tubulin (red), the heart tube (HT) is visible by background fluorescence, arrows indicate the nerve ends. Even when nerves were placed in close proximity (<10μm) to the HT, little outgrowth occurred on the HT or the perineural sheath.
Adult Ganglion and Young HT Co-cultures

In contrast to adult co-cultures, co-culturing adult ganglia with juvenile and embryonic HT resulted in profuse axonal outgrowth on the younger HT as well as extensive growth on the perineural glial sheath (Figure 8) (Harik and Jellies, 1997a; Harik and Jellies, 1997b).

In co-cultures with juvenile HT a dramatic increase in general outgrowth was observed when compared to that of adult co-cultures. All (37/37) preparations exhibited some form of outgrowth, and all but one (36/37) preparation were observed to have robust outgrowth on the perineural sheath of the axons. In more than half (10/16) of the preparations in which the nerve end was in contact with the HT there was robust outgrowth observed on the HT. However, only 2/21 nerves located some distance (20-150 µm) from the HT exhibited outgrowth across the substrate onto the HT (Harik and Jellies, 1997a; Harik and Jellies, 1997b). Minimal outgrowth on HT not in direct contact with nerve ends could be due to the fact that the nerves must traverse the poly-L-lysine. This highly positively charged substrate is not particularly permissive to axonal outgrowth (Chiquet and Acklin, 1986). Patterns of outgrowth support the idea that poly-L-lysine may inhibit the migration of growth cones to the HT as compared to natural leech ECM. The perineural sheath connective tissue however, expresses extracellular matrix molecules that are bound by growth cone receptors that facilitate adhesion and process extension (Chiquet and Acklin, 1986).

In embryonic HT co-cultures results similar to juvenile HT co-cultures were observed with respect to robust general outgrowth. All (41/41) preparations exhibited some outgrowth and most (39/41) had many axons (15-30) that grew on the perineural sheath. 23/27 of the nerves roots in direct contact with the HT extended and grew axons on it (Harik and Jellies, 1997a; Harik and Jellies, 1997b). In contrast to the
Figure 8. Co-cultures of Juvenile HT With Adult Ganglia Stimulate Outgrowth. A) and B) Double labels of axons and juvenile HT using ACT (red) and bis-benzimide (blue). Co-cultures of HT and adult ganglia show profuse outgrowth on the HT and perineural sheath.
adult and juvenile HT, co-cultures embryonic HT at a distance from nerve ends appears to stimulate outgrowth across the substrate to the HT (Figure 9). Almost half (6/14) the observations in which the HT and nerves were not in contact, outgrowth was observed from the cut nerve ends to the HT. These results support the hypothesis that there may be two mechanisms that stimulate axonal outgrowth: (1) a mechanism requiring direct contact of growth cone and target, and (2) a mechanism by which the growth cone responds to diffusible factors, although these experiments can not exclude other possibilities (see discussion). Also, the results of both juvenile and embryonic co-cultures supports the hypothesis that stage-dependent heart-derived cues stimulate axon outgrowth in vitro.

Adult Ganglion and Adult Body Wall Co-Cultures

Body wall muscle was cultured with ganglia to determine whether the target-influences on general axonal outgrowth were target- or age-dependent or both. It was necessary to identify whether axonal outgrowth was a general response to muscle-derived cues or a specific response to cues originating exclusively from the HT.

Sparse outgrowth occurred in adult body wall co-cultures in only four of six preparations. This outgrowth of a few (4-5) axons was limited to the nerve sheath. This response was similar to that seen using the adult HT. Also, all of the nerves in direct contact with the body wall (n=6) failed to extend processes onto it (Figure 10). It had been known however, that adult leech neurons do not regenerate well into the periphery in vivo (Van Essen and Jansen, 1977).

Adult Ganglion and Young Body Wall Co-cultures

All co-cultures with juvenile body wall and adult ganglia resulted in some outgrowth (Figure 11) and 33/38 observations exhibited outgrowth on the perineural
Figure 9. Embryonic HT Stimulates Outgrowth From a Distance. A) and B) Adult axons and embryonic HT. Double labels of adult axons (ACT) and embryonic HT (bis-benzimide) revealed the potential of embryonic tissues to stimulate outgrowth from a distance.
Adult Ganglia Co-cultured With Adult Body Wall Exhibit Limited Outgrowth. Nerves were labeled with anti-acetylated tubulin (red), the body wall with bis-benzimide (blue). Even when nerves were placed in direct contact with the body wall, little outgrowth occurred on the body wall or the perineural sheath. Slightly more than half (10/17) of the nerves in direct contact with the body extended axons onto the body wall. Out of 21 preparations where the tissues were not in direct contact, no outgrowth was observed from the nerve ends to the body wall (Harik and Jellies, 1997a; Harik and Jellies, 1997b).

The axon proliferation of co-cultures with embryonic body wall was similar to those of embryonic HT co-cultures. In almost all (26/27) preparations nerves exhibited general outgrowth, and all but one had outgrowth on the perineural sheath. 10/17 nerves in contact with the body wall extended profuse outgrowth to the body wall. In contrast to the juvenile body wall co-cultures, 3/14 nerves in direct contact with
Figure 11. Co-cultures of Young Body Wall With Adult Ganglia Stimulate Outgrowth. A) and B) are double labels of axons and juvenile body wall (outlined in yellow dots) using ACT (red) and bis-benzimide (blue) showing profuse outgrowth on the body wall and perineural sheath.
embryonic body wall exhibited profuse outgrowth across the substrate and onto the body wall (Harik and Jellies, 1997a; Harik and Jellies, 1997b) (Figure 12). These results also support the hypothesis that stage-dependent body wall-derived cues stimulate axon outgrowth \textit{in vitro}. More specifically these results in conjunction with the embryonic HT co-cultures results support the hypothesis that embryonic cues have the greatest competency to stimulate and support axon outgrowth when compared to older tissue.

Figure 13 summarizes the results of outgrowth from the seven culture situations. All culture arrangements are depicted, including explants in direct contact and separated by some distance.
Figure 12. Co-cultures of Embryonic Body Wall With Adult Ganglia Stimulate Outgrowth From a Distance. A) and B) Adult axons and embryonic body wall. Double labels of adult axons (ACT) and embryonic body wall (bis-benzimide) revealed the potential of embryonic tissues stimulating outgrowth from a distance.
Figure 13. Summary of Outgrowth Results. A indicates adult tissue, J indicates juvenile tissue, and E indicates embryonic tissue. Black lines drawn on explants represent axon outgrowth. In cultures lacking young target tissue there was limited outgrowth from the nerves. In cultures with young target tissue robust outgrowth was observed from the nerves onto the nerve itself and on the target tissue. However, observations suggest embryonic tissue may be more competent to induce outgrowth across the substrate than older juvenile tissue or adult tissue.
CHAPTER IV

DISCUSSION

The purpose of this study was to examine whether target-derived cues stimulate axon outgrowth from neurons in a leech ganglion in vitro. I hypothesized that the expression of target-derived cues is stage-dependent and would stimulate axon outgrowth in vitro.

To test the hypothesis, ganglia and target tissue (HT and body wall muscle) of different ages were co-cultured. These results support the idea of the presence of a target-derived factor that influences axon outgrowth. Furthermore, they provide evidence that supports the hypothesis that the expression of this cue or more than one possible cues is stage dependent. The working hypothesis is that young target tissue expresses this factor more effectively than the older tissue. Previous studies suggest several mechanisms by which target-derived influences may play a role in axon outgrowth and target selection in vivo and in vitro. These influences may be mediated by diffusible factors, adhesion molecules, extracellular matrix, or structural cues.

Substrates can influence outgrowth and target selection by differential interaction of axonal processes with surface antigens or extracellular matrix (Letourneau et al., 1992; Kopp and Jellies, 1994). Many previous studies indicate extracellular matrix molecules as guidance cues. Molecular analysis of guidance cues laminin, LI, TAG-1, N-CAM, conactin and fasciclin II has revealed that they have common structural features belonging to the immunoglobulin supergene families. These multiple Ig-like domains serve a similar function in animals and are conserved in vertebrates and invertebrates (Dodd and Jessel, 1988; Johansen et al., 1994). Leech neuron cultures
have been successfully incorporated into several related studies, such as studying the influences of substrates on growth and synapse formation (Chiquet and Acklin, 1986; Chiquet and Acklin, 1986; Masuda-Nakagawa et al., 1990; F. de Miguel and Vargas, 1997; Fernandez-de-Miguel, 1997).

Absence of Young Target-Derived Cues Results in Limited Outgrowth

In order to test the hypothesis that HT-derived cues stimulated axon outgrowth in vitro, three culture situations were developed: (1) adult HT and adult ganglion co-cultures, (2) adult body wall and adult ganglion co-cultures, and (3) ganglion cultured alone as a control. Very limited axon outgrowth was observed on the nerve sheath or the target in all three situations. These results are supported by early work done on leech neuron regeneration in vivo. Van Essen and Jansen (1977) demonstrated that although leech neurons regenerate well in the CNS, they do so very poorly in the periphery. A possible explanation for limited outgrowth onto adult tissue is that although adult axons form sprouts, the growth cones may be unable to cross from the nerve end to the target (Davies et al., 1997). This idea suggests possibly (1) adult tissue may express molecules inhibitory for the growth of adult axons, or (2) adult tissue is not competent to express growth promoting factors that younger tissues may express. There are precedents for each of these views of inhibition and stage dependency (Davies et al., 1997). An alternative hypothesis is that stage-dependent target derived cues are necessary to stimulate outgrowth. It could be possible that the adult tissue is not capable of expressing the proposed target-derived cues, but younger tissues may be competent to do so.
Stage-Dependent Target-Derived Cues Stimulate Axon Outgrowth In Vitro

In experiments where embryonic and juvenile HT and body wall were co-cultured with adult ganglia robust outgrowth was observed from the cut nerve ends onto the glial nerve sheath and the target tissue. These results support the hypothesis that (1) peripheral cues stimulate general axon outgrowth, and (2) expression of these cues is stage-dependent. A possible explanation for the observed outgrowth may involve matrix expression on the connective tissue nerve sheath, and/or diffusible and contact mediated target-derived factors. Supporting evidence is provided by studies in which suspensions of late embryonic hippocampal tissue were injected with in the myelinated fiber bundles of adult rat (Davies et al., 1997). It was shown that contact with embryonic tissue can induce growth of adult fibers. The author suggests embryonic transplants may utilize several mechanisms to induce outgrowth including, permissive effects of embryonic tissue, adhesion proteins and glial-derived growth factors.

Young Heart Tube

In experiments where juvenile and embryonic HT were co-cultured with adult ganglia an increase in general outgrowth was observed when compared to outgrowth of co-cultures with adult target tissue or cultures with no target tissue. When juvenile HT and adult ganglia were co-cultured in direct contact resulted in robust outgrowth from the cut nerve ends onto the HT surface as well as back onto the glial nerve sheath. However, when the explants were separated very limited outgrowth if any was observed from the nerve end across the poly-L-lysine substrate to the HT.

A possible explanation for outgrowth back onto the nerve sheath rather than across the poly-L-lysine to the target could be due to preferential selection by growth
cones of a permissive substrate. Previous studies demonstrate that the outgrowth of neurites by single identified leech neurons in culture is influenced by the substrate. Chiquet et al. (1988) showed the connective tissue sheath surrounding the CNS expresses a laminin-related leech protein that induces rapid sprouting from leech neurons when cultured together. Masuda-Nakagawa et al. (1990) show that in leeches laminin may also promote axon regeneration at the site of a nerve crush in vitro. Poly-L-lysine, however, is known to inhibit outgrowth in culture (Chiquet et al., 1988) and may account for the observations of very limited outgrowth from nerve ends to a distant target in my experiments.

The results indicate the HT may utilize a contact mediated mechanism that promotes outgrowth onto its self in conjunction with a diffusible cue which promotes general outgrowth onto the nerve. It is not known, however, whether this mechanism involves extra cellular matrix (ECM) or secreted factors. This could be determined by co-culturing fixed HT in direct contact with the nerve. Fixing the tissues will kill the tissue, but preserve some of the 3-dimensional structure of molecules. If ECM is involved in directing outgrowth, then the molecules may still be recognized and bound by the growth cones resulting in outgrowth on the HT. Other studies have demonstrated ECM plays a large role in elaborating axon extensions on targets invertebrate and vertebrate animals. Arber and Caroni, (1995), established thrombospondin-4 (TSP-4) as a neuronal ECM protein associated with certain synapse rich structures. Also, TSP-4 is known to promote neurite outgrowth and may be involved in local signaling in the developing and adult nervous system (Arber and Caroni, 1995). Fas II has also been found to have a profound effect on growth cone contacts. Increase Fas II expression leads to an increase in the number of motoneuron connections in insects (Davis et al., 1997). However, the critical period for the ability of Fas II to influence target innervation is during the period of growth cone exploration.
Binding to certain receptors by ECM components may activate intracellular signal transduction pathways and potentiate mechanisms producing growth factor activity. Adult ganglia in co-culture with embryonic HT exhibited similar outgrowth to the co-cultures with juvenile heart tube. However, in contrast to juvenile HT co-cultures, even when the embryonic HT was not in direct contact with the nerve ends robust outgrowth was observed from the adult nerves across the substrate onto the HT. These results indicate the embryonic HT may express a diffusible influence over the axons strong enough to overcome the non-permissive poly-L-lysine substrate and induce directed outgrowth to its self. Once the axons contact the target outgrowth may be mediated by surface bound contact mediated cues. This may be similar to the HE outgrowth during development in vivo, in that the HE axon must be directed to grow approximately 100µm to contact the location of the future HT. Once the HT is contacted the HE then extends many terminals on the HT surface. An alternative not excluded is that cells or secreted factors from the embryonic explant bridge the gap and mask the poly-L-lysine.

There exists a great body of evidence supporting target-derived chemotactic signals in axon guidance and for appropriate innervation. For example, HE’s in segments deprived of HT in ablation experiments, mentioned above, will extend (in animals 2-4 months survival) to make close contact with the cut ends of HT 2-3 segments away (Jellies, 1995). In co-culture experiments using vertebrate neurons (Kuffler, 1996, Perez et al., 1997, and Xie et al., 1997) substrate bound and diffusible factors were utilized to demonstrate that growth cones turn and migrate up concentration gradients of peripheral target-derived factors in vitro. Early experimental manipulations (Lance-Jones and Landmesser, 1980; Lance-Jones and Landmesser, 1981) altering the relationship between chick motoneurons within the lumbosacral
motor column and their targets in the limb demonstrate axons alter their course to specifically innervate their correct target.

Results from embryonic HT in conjunction with juvenile HT co-cultures argue that more than one mechanism may be employed to stimulate outgrowth of leech neurons in vitro. Also, there may be a developmental window of opportunity, a critical period, for these mechanisms to take place. Embryonic tissue may in fact be competent to express a cue stronger than, or in addition to that of juvenile HT to induce outgrowth over the non-permissive poly-L-lysine substrate. These results also demonstrate that the adult neurons are capable of responding to target-derived cues.

**Young Body Wall**

Juvenile body wall also stimulated greater outgrowth from adult neurons when compared to co-cultures with adult body wall in vitro. When the nerve ends were in direct contact with the body wall robust outgrowth was observed onto the nerve sheath and onto the body wall. However, similar to the juvenile HT co-cultures, when the body wall was some distance from the nerve end, there was outgrowth on the nerve sheath, but axons did not extend upon the poly-L-lysine substrate to the body wall. The molecules expressed on the glial nerve sheath may provide a more permissive substrate for growth cone extension than the non-permissive poly-L-lysine. Thus, when given a choice of two substrates, one permissive and the other non-permissive, growth cones may exhibit preferential binding. Many more axons may extend upon the nerves rather than on the poly-L-lysine to a target. Embryonic body wall muscle also stimulated much outgrowth on the nerve sheath and upon itself when explants were in direct contact. Also, similar to embryonic HT, embryonic body wall was capable of inducing outgrowth up to 150µm across the poly-L-lysine upon its self.
Although, in this leech species the proper final target of the HE is the HT, during pathfinding and target selection the HE contacts body wall muscle before localizing all terminals to the HT. This may provide an explanation for the outgrowth onto the embryonic body wall in vitro. The body wall may be competent to express the same factors in vitro to promote outgrowth as in vivo during embryogenesis. Also, as with the HT co-cultures, the identity of the axons extending upon the body wall are not known, nor is the presence of functional synapses.

Neurons may differ in how they form synapses in vitro, and may employ different mechanisms of synaptogenesis. For example, some neurons such as the B19 motoneuron in Heliosoma, when given a choice of possible targets will selectively form chemical connections with its appropriate target (Zoran et al., 1990; Zoran and Poyer, 1996). Other cells in Heliosoma form nonspecific synaptic connections with inappropriate targets in vitro. The goals of future studies related to my project address, (a) determining the identity of any neurons that may re-innervate target tissue, (b) examine the specificity of re-innervation in vitro, (c) selectivity of HE outgrowth and possible synaptogenesis in culture, and (d) quantifying axon outgrowth.

Future Directions

Although extensive or elaborate identifications of the axons elaborating on the HT and body wall are beyond the scope of this project, a number of major established methods could be employed to begin to gain insight into the issue. Possible methods to be explored for doing so will incorporate electrophysiology, Dil injections in the HT, intracellular dye filling of the HE and immunohistochemistry directed to the HE. Quantification of outgrowth may also be explored with image analysis. Digital images could be analyzed by using the NIH-Image software. The area of outgrowth could be traced and converted to area (Huang et al., 1997; Zipser and Cole, 1991) normalized to
non-fluorescent area. Although the present study robust and general outgrowth responses, more refined conditions of purified materials might exhibit more subtle responses requiring quantification of outgrowth.

Although robust general outgrowth was observed upon the young HT and body wall tissue, it was not determined whether any of these axons formed functional synapses with the target tissue. Electrophysiology could be used to test hypotheses regarding the specificity of the target-derived cues to induce formation of functional synapses \textit{in vitro}. One could determine if the HE forms functional synapses on the target by utilizing a glass pipette microelectrode to pass current into the HE and a second electrode impaling the muscle fiber alleged to be innervated by the HE. If a current passed into the HE induces a postsynaptic potential change in the muscle, then one could conclude the HE is capable of reinnervating a target \textit{in vitro}. This technique could be used to determine if the HE specifically reinnervates the HT only, or also reinnervates the body wall \textit{in vitro}. Also this technique could be repeated with other neurons in the cultured ganglia to determine the specificity of the target-derived cues. If the HE were the only neuron found to form functional synapses that would support the hypothesis that the target-derived cues are capable of specifically inducing the HE to reinnervate its target \textit{in vitro}. These results would also support the idea of HT-derived factors as cues in HE pathfinding and target selection \textit{in vivo}. In conjunction with electrophysiology, Dil injections of HT's in co-culture could be performed to examine any possible re-innervation of the HT. As mentioned before (material and methods) Dil is a lipophilic dye. Therefore, the dye is transported from the HT lumen through the synapses of neuromuscular junctions and will retrogradely label any neurons which directly form functional synapses on the HT.

Several fluorescent dyes such as Lucifer yellow (LY), biotin dextran, and horse radish peroxidase (HRP) have been successfully used in single cell dye fills to visualize
cell morphology and neuron projections (Jellies and Kristan, 1991; Jellies et al., 1992; Johansen et al., 1994; Kopp and Jellies, 1994; Jellies and Kopp, 1995; Jellies et al., 1996b). Biotin dextran is a physiologically inert dye that could be a useful tool in identifying the HE processes in culture. Dextran could be injected in the HE while still in the animal. Following, the injection the ganglion and target tissues would be co-cultured and processed as the above methods (materials and methods) and assayed for outgrowth. The dye will selectively label the HE and its axon. Therefore, one could visualize if the HE axon is one of those capable of growing upon the HT, body wall, or glial nerve sheath in culture. LY and HRP could also be used in a similar fashion. Likewise, neuropeptide antibodies may prove useful in this regard. In particular, FMRFamide neuropeptides (peptides with the carboxyl terminal sequence Arg-Phe-amide) have been identified in several species throughout the animal phyla (Evans et al., 1991). In the leech this neuropeptide is found in several neurons including the HE among others, as well as the HE processes which innervate the heart (Kuhlman et al., 1985; Wenning et al., 1993) and is used to modulate the activity of the HT (Kuhlman et al., 1985; Evans et al., 1991). Immunohistochemical localization of FMRF-amide-like substances could also be used to identify the HE in culture.

Additional studies are needed to make conclusions regarding the specificity of re-innervation, selectivity of HE outgrowth or the identity of other neurons exhibiting outgrowth. The present results however, support the hypothesis that stage-dependent target-derived factors can effectively stimulate axon outgrowth in vitro. Also, they argue that the adult neurons remain competent to respond to target-derived factors, but that muscles like HT may lose its ability to express or present these factors. Furthermore, determining the specificity of the heart-derived cues, axon outgrowth, and synapse functionality will be a major step in elucidating the specific role, mechanism and identity of the heart-derived cues.
Appendix A

Materials and Methods and Results From Single Cell Cultures
Materials and Methods

Cultures of dissociated *Hirudo* neurons were generated (Chiquet and Acklin, 1986; Chiquet *et al.*, 1988; Masuda-Nakagawa *et al.*, 1990; Acklin and Nicholls, 1990; Fernandez-De-Miguel, 1997; F. de-Miguel, and Vargas, 1997) as the most direct approach to examine the influences of HT-derived cues on the HE outgrowth *in vitro*. Although many different substrates and conditions were tried, this technique resulted in complications with cell adhesion and HE identification and were deemed promising but not adequate for a graduate project. Concanavilin A, a plant lectin, Leech extracellular matrix, and HT matrix all used in previous leech neuron cultures failed to cause adherence of neurons or HT cells sufficiently to carry out *in vitro* studies of outgrowth. However, the single cell cultures did serve a purpose in determining if ACT could reliably label neurites in culture.

Mid-body ganglia (except those from segments 5 and 6) were microdissected from adult animals and pinned ventral side down in sterile saline filled plastic culture dishes (Falcon, 35x10mm) coated with Sylgard (Dow Corning). Ganglia were transferred to Leibovitz L-15 medium (Sigma) supplemented with 6mg glucose/ml (Sigma) and 6 µg glutamine/ml (Sigma), 2mg/ml protease (Sigma, from *Streptomyces griseus*) and 10% Penicillin-streptomycin (pen-strep) (Cellgro/Sigma). The connective tissue capsules surrounding the ganglia were removed with forceps and the ganglia were incubated for 1 hour at room temperature. Cells from the anterolateral packet of the ganglia were removed by aspirating them into a glass micropipette (Flaming/Brown micropipette puller, model P-87) (Fig. 14). Cells were plated on plastic (Thermanox) coverslips in L-15 in plastic culture dishes. Cultures were maintained for 5 to 10 days. Cultures were fixed in cold 4% paraformaldehyde overnight and processed for immunocytochemistry with primary antibody anti-acetylated tubulin to visualize any
neurite outgrowth.

![Diagram of Dissociating Neurons. Single neurons were removed from the anterolateral packet by aspiration into a micropipette. Neurons were then transferred to a culture dish and plated.]

**Figure 14.** Diagram of Dissociating Neurons. Single neurons were removed from the anterolateral packet by aspiration into a micropipette. Neurons were then transferred to a culture dish and plated.

**Results**

Despite difficulties with certain aspects of the neuron cultures, I was able to examine if a mAb directed against ACT would label individual neurites in culture and if the label could be visualized with an epifluorescent microscope. Tubulin antibodies have been used to investigate the axonal projections of vertebrate neurons (Jellies et al., 1996). Tubulin antibodies also label all known central neurons and their processes in Hirudo medicinalis (Jellies et al., 1996).

DIC optics were used in conjunction with the ACT antibodies to confirm that the antibodies labeled the central neurons and their processes. All cells that survived incubation and their processes were observed to be labeled with the ACT antibodies.
These results indicate that ACT antibodies could reliably be used in tissue co-cultures to assay for axonal outgrowth.

In addition, these cultures confirmed the media preparation for cell survival and the feasibility of isolating individual neurons in future studies.


