UNIVERSITY OF CALIFORNIA, SAN DIEGO

# Effects on motor neuron development of altering peripheral targets in embryonic leeches (*Hirudo verbana*)

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

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Gina Sosinsky

2009

The Thesis of Sandahl Hygeia Nelson is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego
2009

# TABLE OF CONTENTS

# LIST OF FIGURES

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ABSTRACT OF THE THESIS

Effects on motor neuron development of altering peripheral targets in embryonic leeches (*Hirudo verbana*)

by
Sandahl Hygeia Nelson
Master of Science in Biology

University of California, San Diego, 2009

Kathleen French, Chair

During embryonic development, axons that project into the peripheral tissue encounter many different cues that can affect their final phenotype. We have studied the development of individually identified motor neurons in medicinal leeches (*Hirudo verbana*). We hypothesized that contact with peripheral targets provide signals that direct the formation of central synaptic connections. To test this hypothesis, we transplanted pieces of body wall into ectopic locations to ask if the central connections of motor neurons change when they contact the "wrong" peripheral target. We used embryos at 47-50% of development, a stage at which neurons are just beginning to form their central electrical connections. We transplanted tissue from a donor embryo and implanted it into the opposite region of a host embryo. We then let our embryos develop to a juvenile stage. External pigment patterns indicated that transplanted tissues retained

their original fate. Immunostaining for acetylated tubulin revealed that the transplanted tissue became innervated, although less densely than un-manipulated tissue. Injections of AlexaFlour 488 dextran into motor neurons adjacent to an ectopic transplant indicated that the axons of these neurons projected into the periphery similarly to unaltered controls. Injecting Alexa Flour 488 plus Neurobiotin, which crosses gap junctions, revealed no statistically significant difference in the number of cells that were dye-coupled to the filled cell in embryos that received ectopic transplants, although the neurons and connections within the ganglion become markedly un-patterned, suggesting that disrupting the periphery exerts at least some influence in the central nervous system.

# INTRODUCTION

During embryonic development, axons that grow into peripheral tissue encounter a variety of cues that may affect their final phenotype. Many studies in both invertebrates and vertebrates have shown that peripheral cues can play an important role in shaping the central nervous system. For example, in the bullfrog tadpole (Smith and Frank, 1987) when thoracic dorsal root ganglia (which contain the somata of sensory neurons that innervate the skin of the trunk) were transplanted to the brachial level, the normal location of the forelimb afferents, the transplanted thoracic sensory neurons innervated and projected appropriately for their novel brachial location. HRP labeling of the central projections revealed that these transplanted sensory neurons also formed central connections appropriate to their novel peripheral target, rather than to their location of origin. Similar results have been seen in the chick. Muscle sensory neurons that normally would contact ventral tissues were forced to contact dorsal muscle. Although these axons followed typical ventral peripheral pathways, when these sensory neurons formed synapses onto dorsal muscle fibers they also formed central synaptic connections appropriate for their new dorsal target muscle, rather than for their originally fated ventral target (Wenner and Frank., 1995). These results are consistent with the idea that the connections between neurons and their targets can be important in determining central connections.

In addition, peripheral contacts can affect neuronal survival, and recent work suggests a potential mechanism for this effect. Ablating a limb bud from a chick embryo

at embryonic day 8 (Hamburger and Hamilton stage 17/18) (Hamburger and Hamilton 1951) eliminated 90% of tyrosine receptor kinase C-positive (TrkC+) sensory neurons of the dorsal root ganglia that would normally supply muscle spindles in the ablated limb, but the exogenous application at stage 25 of neurotrophin-3 (NT3) derived from limb-buds restored to normal the population of TrkC+ neurons. The rescued TrkC+ neurons developed morphologically normal central projections, suggesting that peripheral factors play an important role in the survival of central neurons. Applying exogenous NT3 increased the number of TrkC+ neurons in control embryos in which all limb buds were intact (Oakley et. al., 1997).

The developmental effects of contacts between neurons and their usual targets have also been studied in the medicinal leech, *Hirudo verbana* (until recently classified with *Hirudo medicinalis*) (Sidall, et al 2007) Leeches are annelid worms; that is they have a segmented body plan (Fig 1 c, d). Each of the 21 mid-body segments between the head and the tail contains a ganglion, and each ganglion contains the somata of about 400 neurons that exert considerable local control over that segment (Fig 1 a,b). Ganglia are connected with one another by way of connective nerves. Two of the midbody segments are somewhat different: leeches are simultaneous hermaphrodites, and in *Hirudo* the fifth midbody segment contains the male reproductive ducts (the testes are distributed in the posterior body), and the sixth midbody segment contains the female reproductive system. In leech embryos, disrupting the connection between the periphery and the neurons that have somata in the fifth or sixth midbody ganglia affects the developmental fates of at from least some neurons in those two ganglia. When the nerve roots through which axons

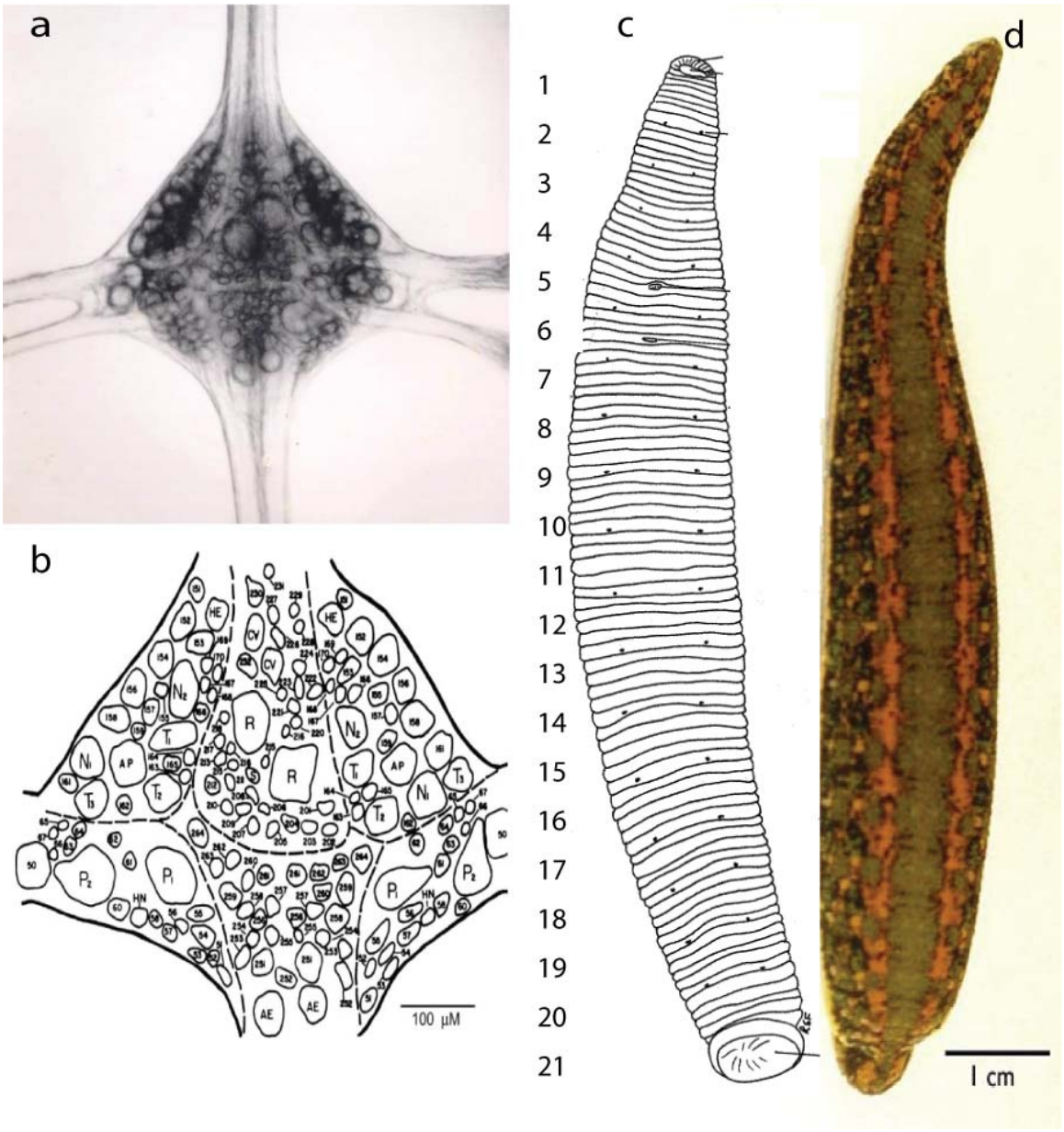



**Figure 1. Leech Introduction. a)** Adult leech ganglia showing anterior and posterior connectives as well as lateral nerves which connect the ganglion to the body wall (photo by P. Katz). **b)** Ventral side of the adult leech ganglia (photo P. Katz) **c)** Sketch of adult leech showing the 21 mid-body segments as well at the head and tail segments (sketch by R. Fox) **d)** Dorsal side of adult leech *Hirudo verbana*.

these two segments project to the peripheral embryonic sex organs are severed, neurogenesis of a population of small neurons that are unique to these two ganglia (the peripherally-induced central, or PIC, neurons) is prevented (Baptista et. al., 1990).

The function of PIC neurons remains unknown, but peripheral contact also affects the development of neurons that have been better studied. Each ganglion in the leech central nervous system (CNS) contains a pair of large, serotonergic Retzius (Rz) neurons. The serotonin released centrally by these neurons plays an important role in controlling the activity level of a leech (Willard, 1981) Rz neurons are found as two phenotypes: in most body segments Rz neurons branch diffusely among the muscles of the body wall and just under the skin (Lent, 1977). We call these neurons "standard Rz neurons." However, in the two reproductive segments, the Rz neurons arborize densely in the muscular walls of the reproductive ducts and do not innervate the body wall (Jellies, et al., 1987). In fact, several properties of Rz neurons depend on the nature of the peripheral tissue that they contact during a narrow developmental window; these properties include central morphology, projection pattern out of the ganglion, and synaptic contacts (Jellies, et al, 1987). When the embryonic reproductive ducts were ablated about half way through embryogenesis, the Rz neurons in segments 5 and 6 developed morphology and synaptic inputs similar to standard Rz cells (Loer, et al., 1987; Loer and Kristan 1989) .This result suggests that the peripheral contacts made by Rz neurons affected both their morphology and their synaptogenesis within the CNS; the mechanism of this effect has yet to be characterized.

Rz neurons are not the only neurons in the leech that respond to a change in cellular contacts during development. For example, contact between the two AP neurons in each ganglion forces each of them to project to only one side of the body wall, but if one of the AP neurons is ablated during embryogenesis, the survivor projects to both sides of the body (Wolszon, 1995).

Previous work in our lab revealed that cutting the nerve roots on one side of a ganglion was followed by exuberant overgrowth of the processes of some--but not all--motor neurons that normally project to the periphery through those roots (Johnson, et al., 2000). Normally each motor neuron of a leech innervates muscle fibers within a specific region of the body wall, and these peripheral contacts are established before central connections develop (Johnson, et al., 2000). Following up on the results described in Johnson, et al., 2000, the current study was designed to determine whether altering the peripheral target of identified motor neurons in embryonic leeches would alter central morphology and synaptogenesis. To force neurons to innervate novel targets in embryos, we surgically transplanted dorsal tissue into a ventral position where axons would encounter it as they grew out of a ganglion. As a result, motor neurons that normally would have innervated the ventral body wall would instead contact dorsal muscle tissue, the "wrong" target. We then assayed for several properties of specific identified motor neurons.

## METHODS

### *Animals*

Adult medicinal leeches (*Hirudo verbana*) were purchased from a commercial supplier [Leeches USA (Westbury, NY) or Carolina Biological Supply (Burlington, NC)] and maintained in a breeding colony in our laboratory. Embryos were released from their cocoons no earlier than 9 d after egg deposition and were then held at 20–24°C in "embryo water," that is, sterile-filtered Arrowhead Spring Water (Arrowhead Water, Brea, CA) with 32 μmol of $MgCl_2$ and 40 μmol of $CaCl_2$ added per liter."

### *Staging*

We determined the embryonic stage of each embryo based on external morphological features; stages run from egg deposition, or 0% of embryonic development (% ED) to 100% ED (Reynolds et al., 1998a). All surgery was performed at 47.5-49% ED (Fig. 1a) because previous work in the lab showed that the excitatory motor neuron dorsal exciter 3 (DE-3) begins to form electrical synapses with other neurons no earlier than this stage (Marin-Burgin, et al., 2005).

### *Transplant surgery*

Embryos were anesthetized in ice-cold leech saline, which contains (in mM): 115 NaCl, 4 KCl, 1.8 $CaCl_2$, 1.5 $MgCl_2$, 10 dextrose, 4.6 Tris maleate, and 5.4 Tris base, pH 7.4, and to which ethanol was added to a final concentration of 8% (v/v). Anaesthetized embryos were placed in rectangular chambers cut into Sylgard-filled plastic Petri dishes and were restrained with two latex strips, (cut from a latex glove) that crossed the

embryo's body and were pinned to the Sylgard on either side of the embryo. Two embryos, a host and a donor, were immobilized next to each other.

In dorsal-to-ventral ectopic transplants, a rectangle of dorsal tissue was transplanted into a ventral position adjacent to a midbody ganglion. To prepare the host embryo, a square of ventral tissue was removed (Fig. 2 b), with the anterior boundary of the square being the posterior margin of the ganglion in the segment just anterior to the cut segment and the posterior boundary of the square being the anterior boundary of the next posterior ganglion. The medial boundary of the cut was the ventral midline of the embryo and the lateral boundary was the middle of the nephridium ipsilateral to the cut, which is approximately the ventral-dorsal boundary (Johnson, et al., 2000). All tissue was taken from locations between segments 8 and 13. Next a square of tissue was cut from the donor embryo in the same segment into which it would be transplanted and using the same anterior and posterior boundaries as in the host. In embryos receiving a ventral to dorsal (V→D) ectopic transplant, a rectangular piece of ventral body wall was implanted into a hole at the edge of the germinal plate (i.e., the future dorsal territory). In ectopic transplants the medial boundary was the middle of the nephridium (the primordial lateral edge of the leech), and the lateral boundary was the edge of the germinal plate, which is the embryonic dorsal midline (Fig. 2b). In eutopic transplants, a square was cut from the donor with the same boundaries as the tissue that was removed from the host (Fig. 2c). The donor tissue was then moved into the open body wall of the host embryo, taking care to maintain the anterior-posterior orientation. This tissue was covered with a small shard of broken cover slip to hold the transplanted tissue in place, and the recipient was allowed

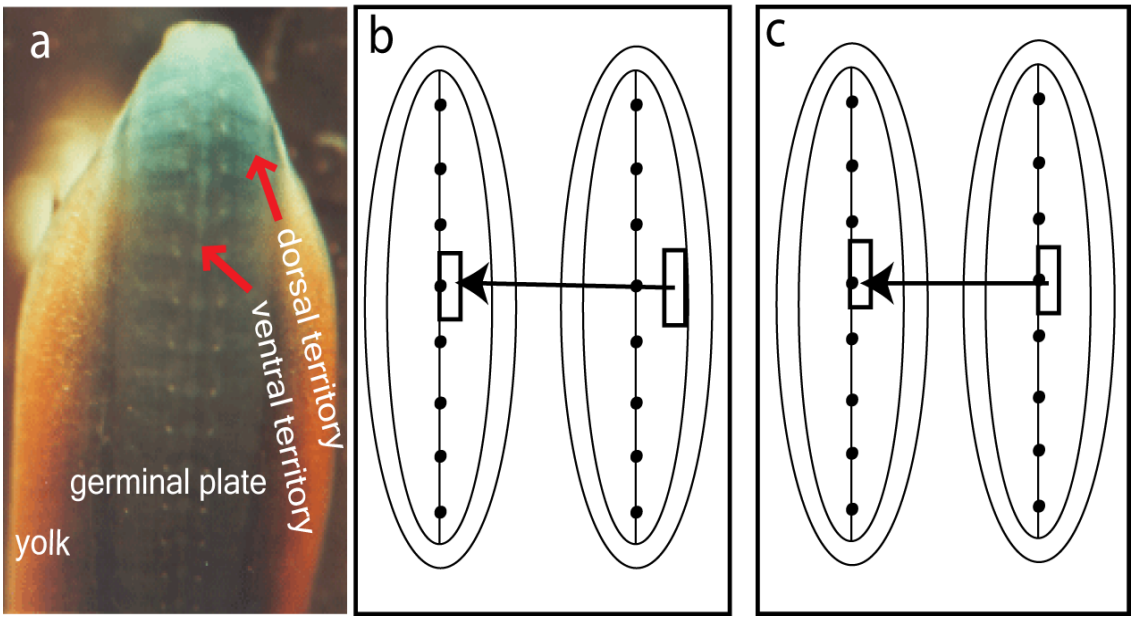



**Figure 2. Transplanting methods. a)** Intact leech embryo at about 50% of embryonic development (50% ED). The embryo proper (germinal plate) develops on the surface of a yolk sac (yolk). Development proceeds laterally from the ventral midline and posteriorly from the head (at the top of this image). Because the germinal plate grows around the yolk sac, and the edges fuse to form the dorsal midline, at this stage, the two edges of the germinal plate together are the presumptive dorsal midline. Fast green was injected under the embryo to increase contrast (photo by K. French). **b)** In ectopic transplants, about 2 segment equivalents of dorsal tissue were removed from a donor embryo at approximately 50% ED and placed into the same anterior-posterior position in the ventral surface of the host, just lateral to the ventral nerve cord. **c)** In eutopic transplants, a piece of ventral body wall was removed from a donor embryo at approximately 50% ED and placed in the same anterior-posterior position in the ventral surface of the host, just lateral to the ventral nerve cord.

to heal. After 45 minutes, most of the ethanol-saline solution was suctioned off and replaced with embryo water. The embryos were then allowed to heal for another 1-2 hours. The cover slip bandage was then removed, and the embryo was released from the latex restraints and moved into a fresh Petri dish containing embryo water. All leeches were allowed to develop past 100% ED, and the results of the transplants were analyzed. Visual observation confirmed that the transplanted tissue had healed into the body wall.

*Intracellular injections for visualizing projection patterns*

Juvenile leeches were anesthetized in 8% ethanol/leech saline, the nerve cord was exposed, and individual motor neurons were impaled with sharp microelectrodes filled with 2.5% lyseinated Alexa Fluor 488 dextran, 10,000 MW, (Invitrogen, Carlsbad, CA) dissolved in de-ionized water. Embryos were viewed through a compound microscope equipped with fluorescence optics. Dye was passed into the cell using a sine-wave current protocol with a 1 second cycle period and a maximum current of +0.5–+2 nA. In some embryos, a diffusion time of 45 to 60 minutes was allowed; then the living preparation was imaged using a Zeiss laser-scanning confocal microscope controlled by Bio-Rad software (Hercules, CA, USA) to determine the extent of that neuron's growth into the transplant region. Other leeches were processed further as described below.

*Immunohistochemistry*

Dissected juvenile leeches, with or without dye-filled cells, were fixed overnight in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 5˚C. Preparations were then rinsed with PBS 6 times, 15 minutes each, before further processing. Axons were labeled both centrally and peripherally using a monoclonal antibody (mAb) directed

against acetylated tubulin (ACT; Sigma) and a protocol previously described by Jellies et al., 1996. Briefly, the primary Ab was used at a dilution of 1:1,000 in 10% goat serum, and 1% Triton X-100 in PBS. Incubations were carried out overnight in a refrigerator at 5˚ C. After 6 washes in PBS, 15 minutes each, the tissue was incubated in Alexa Fluor 568-conjugated rabbit-anti mouse IgG at a dilution of 1:500 in 10% goat serum and 1% Triton X-100 in PBS and was incubated overnight at 5˚C . The following day it was washed with PBS, 6 changes, 15 minutes each. The tissue was then dehydrated though an ethanol series, cleared with methyl salicylate, and mounted in Gurr DePeX mounting medium. These leeches were viewed using the Zeiss laser scanning confocal microscope controlled by Bio-Rad software (Hercules, CA, USA).

*Quantification of ACT staining*

The average and standard deviation (SD) of the pixel intensity across each collapsed confocal Z stack was determined, and threshold value was set at the average pixel intensity across the merged stack plus 1 SD. To compensate for differences in animal size, in the periphery adjacent to the ganglion within the segment containing the transplanted tissue (or control tissue) we drew a region of interest (ROI) that was equal to 4 times the area of the ganglion in that segment. We then applied the threshold to the region of interest (ROI) and determined the percentage of pixels within the ROI whose intensity exceeded the threshold, that is, the percentage of the ROI with positive ACT staining.

*Inracellular injections to reveal dye-coupling*

Dye coupling was assayed using the technique described in Marin-Burgin et. al., 2006.

Briefly, juvenile leeches were anesthetized in 8% ethanol-leech saline, the nerve cord was exposed, and individual neurons were impaled with sharp electrodes filled with a combination of 2.5% (wt./vol.) Neurobiotin (Vector Laboratories, Burlingame, CA, USA) and 2.5% lyseinated Alexa Fluor 488 dextran (10,000 MW) (Molecular Probes, Eugene, OR) dissolved in de-ionized water. Dye was passed with sine waves of positive current (0.5–2 nA) using the same protocol as above. The dyes were allowed to diffuse for 60 min, after which the tissue was fixed with 2% paraformaldehyde in 0.1 M sodium phosphate buffer (ph 7.4) overnight at 5˚C. The fixed ganglia were then rinsed with PBS and permeabilized with 0.3% Triton X-100 in PBS (PBX), followed by an overnight incubation in Cy3-conjugated Streptavidin at 2 μg/ml in PBX (Jackson Immuno Research, West Grove, PA). The tissue was thoroughly rinsed in PBS, dehydrated though an ethanol series, and cleared with methyl salicylate. Tissue was mounted in Gurr DePeX mounting medium (Electron Microscopy Sciences, Hatfield, PA, USA) and imaged with a Zeiss laser scanning confocal microscope using Bio-Rad software (Hercules, CA, USA).

RESULTS

The goal of these experiments was to force embryonic motor neurons to grow into muscles that they would not normally encounter in order to determine how contact with novel targets might affect central connections.

*Tissue retained its original fate after transplantation.*

Both parts of the project required the transplanted tissue to retain its original fate, rather then to take on the fate of the surrounding host tissue. Transplant surgeries were performed on embryos at 48%ED, and at this stage, dorsal and ventral skin lacks pigmentation and thus the two regions look the same. To determine whether transplanted tissue retained its original fate after embryonic transplantation we allowed the leeches to develop to a juvenile stage (at least 18 days later). By this stage, secondary body pigment had arisen, and a simple visual check indicated that the tissue had not changed its original fate (Fig. 3a, c). In addition, the pigmentation, or lack of pigment in the case of V→D transplants, indicated the boundaries of the transplanted tissue and confirmed that the transplanted tissue had not been rejected. In eutopic transplants, the heart tube, typically located near the lateral edge, but in ventral territory, differentiated in the transplanted tissue, but it typically failed to connect with the heart tube in the host's own tissue. The discontinuity in the heart tube indicated the anterior and posterior boundaries of the transplanted tissue and confirmed that the transplant had not been rejected (Fig 3 b,d).

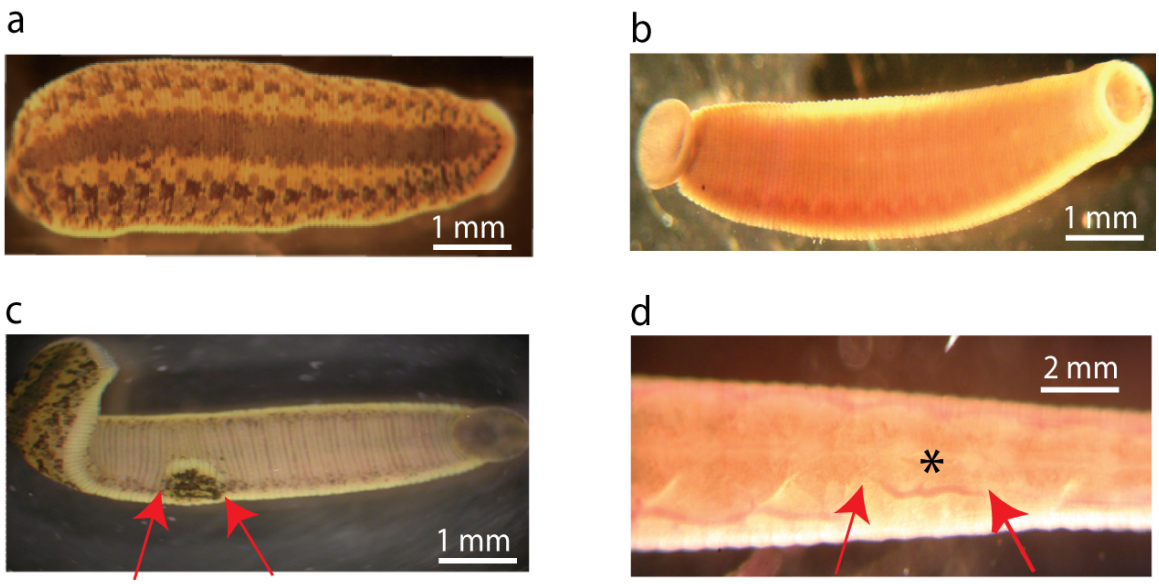


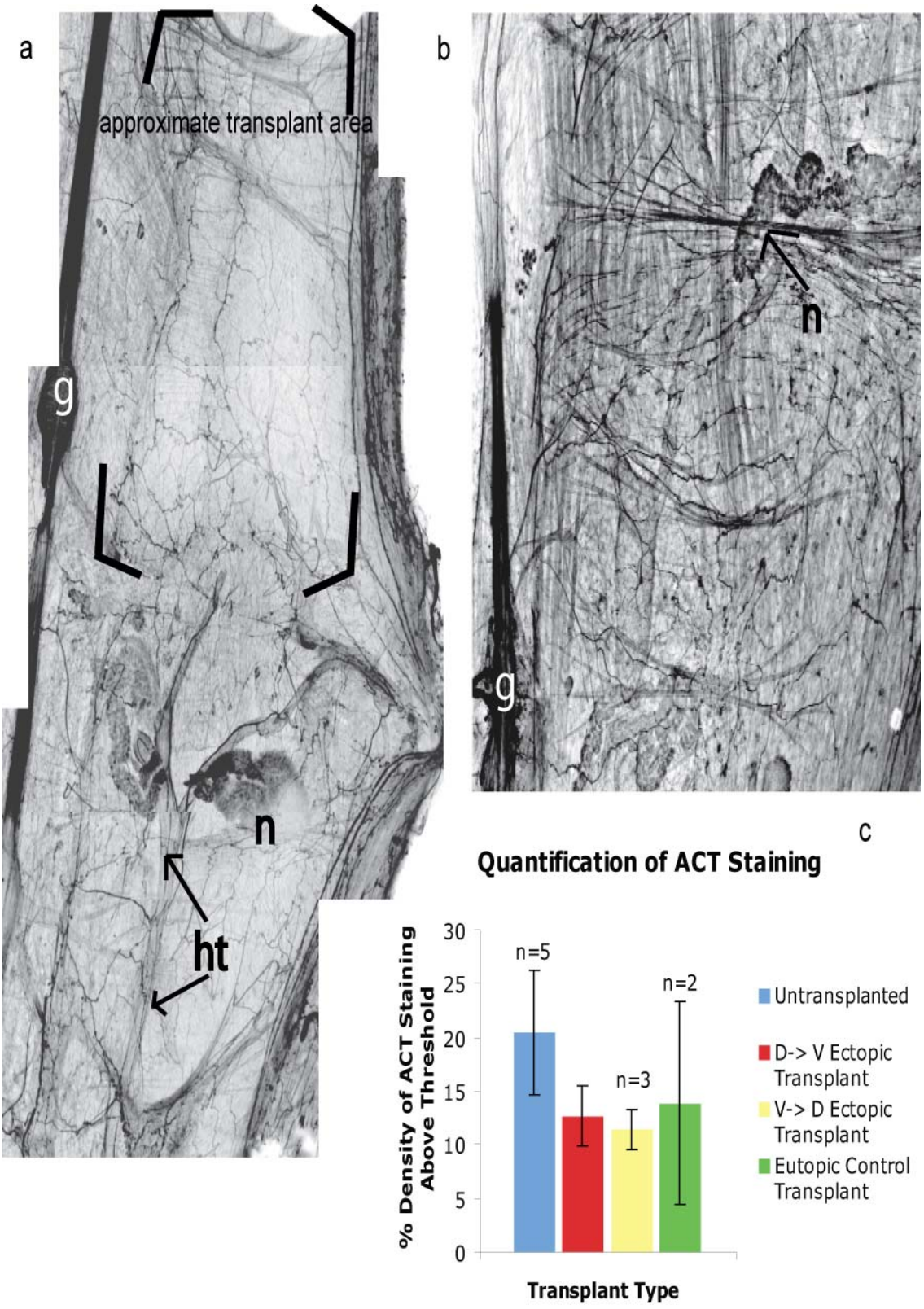

**Figure 3. External appearance of control and experimental embryos at the juvenile stage. a)** Dorsal surface of an intact juvenile leech. **b)** Ventral surface of an intact juvenile leech. **c)** Ventral surface of a juvenile leech following ectopic transplant of dorsal body wall into a ventral position at 50% ED. The dark pigment seen at the site of the transplant (red arrows) suggests that the transplanted body wall retained its dorsal positional identity following the transplant surgery. **d)** Ventral surface of a juvenile leech following eutopic transplant into a ventral position at 50% ED. The heart tube is displaced on the side of the transplant (*), but the transplanted tissue retained typical ventral pigmentation and morphology. Small arrows indicate anterior and posterior boundaries of transplanted tissue.

*Transplanted tissues became innervated.*

Because one goal of our research was to determine how central connectivity differed if "incorrect" peripheral targets were encountered, it was important to determine whether transplanted tissue became innervated. To assay for peripheral innervation, we used an antibody that specifically binds to the cytoskeletal component acetylated tubulin (ACT), which in leech neurons is found primarily in peripheral axons (Jellies et. al., 1996) . Antibody labeling was carried out in leeches that had reached the juvenile stage, always at least 3 weeks after the initial transplants. Only embryos that were judged, based on external appearance, to have retained the transplanted tissue were used ; the boundaries of the transplant were identified based on pigmentation in the skin and other structural characteristics. In host embryos that received ectopic transplants, or eutopic transplants of ventral tissue, axons labeled by the anti-ACT antibody were regularly seen to invade the transplanted tissue (Fig. 4 a,b,c).

We quantified this ACT staining to evaluate innervation. To quantify this staining we took a stratified random sample, grouped based on transplant type (ectopic, eutopic, or control), in order to evaluate 14 segments in 14 different embryos. This assay confirmed that both ectopic and eutopic transplanted tissue was innervated, but the density of staining was lower than in the equivalent region of body wall in unmanipulated embryos. ANOVA analysis showed that this difference was not significant. (Fig. 4 d)

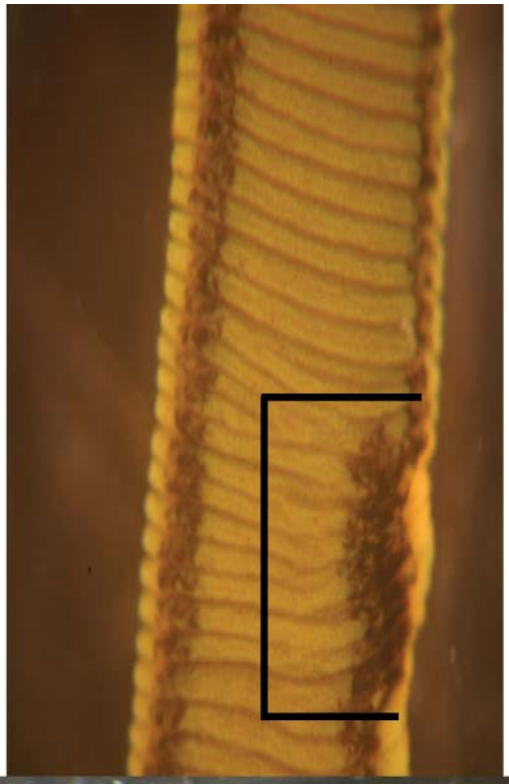
**Figure 4. Innervation pattern in the body wall of juvenile leeches as revealed by an antibody to acetylated tubulin**. g= Ganglion, ht= Heart Tube, n= Nephridia **a)** Innervation pattern in two adjacent segments. The segment at the top of the figure contains ectopically transplanted dorsal tissue. The black "corners" indicate the approximate boundaries of the transplant. The bottom of the figure shows the body wall in the adjacent segment and beyond the posterior boundary of the transplanted tissue. **b)** Innervation pattern in a section containing only eutopically transplanted ventral tissue. **c)** Quantification of ACT staining in intact embryos (blue), embryos that received dorsal to ventral transplants (red), ventral to dorsal transplants (yellow), or ventral to ventral transplants (green).


a
approximate transplant area
g
n
ht
b
n
g
c
Quantification of ACT Staining
% Density of ACT Staining Above Threshold
30
25
20
15
10
5
0
n=5
n=3
n=2
Transplant Type
Untransplanted
D-> V Ectopic Transplant
V-> D Ectopic Transplant
Eutopic Control Transplant

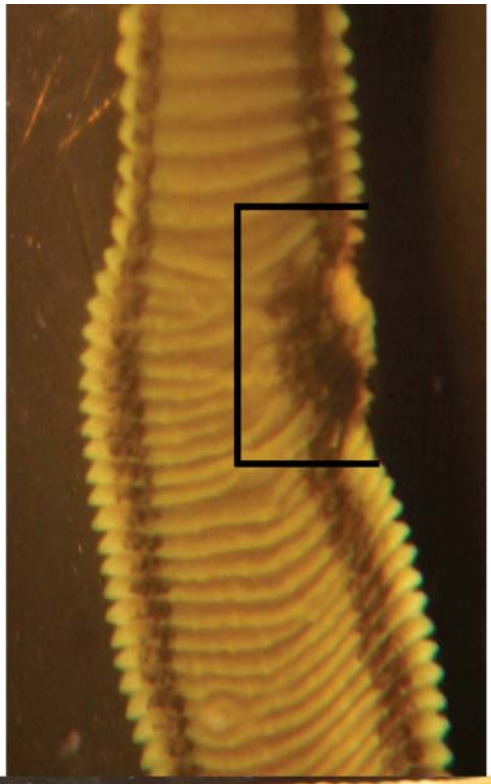
Although ACT labeling indicated that axons had extended into the transplanted ectopic tissue, it could not assay for functional synapses. We used a behavioral assay to test for functional synapses. Most mid-body segments of a leech consist of five rings called annuli; each annulus is supplied with a set of tiny anterior-posterior muscles that, when contracted, cause the annulus to raise up and give the outer surface of the leech a corrugated appearance. These little muscles are called "annulus erector" muscles, and they are controlled by two motor neurons (a bilateral pair) in each segment. The annulus erector neurons in each ganglion control the muscles in only the segment containing the ganglion, so the reflex provides an assay for functional local synapses. Although a variety of stimuli can activate the annulus erector muscles, one reliable method is to transfer a leech to 8% ethanol in normal leech saline. We used this response to test for functionally active synapses in the transplanted tissue. When juvenile leeches that had received transplants (ectopic or eutopic) were put into 8% ethanol, the annuli in the transplanted tissue responded to an extent equal to the surrounding native tissue (Fig. 5 a,b,c,d). The question arises as to whether the EtOH is causing a stimulation of the annulus erector muscles directly or via stimulation of the AE neuron. Because this response is only when a leech is fist put in the 8% EtOH and is not yet anesthetized, and it disappears after the leech is not anesthetized, we hypothesized that the EtOH was acting on the AE neuron rather then directly on the AE muscles. To test this hypothesis we dissected a juvenile leech, denervating one section. We placed this leech in 8% EtOH to see if the response was still present. We found that annulus erection was absent in the denervated section, but was present in the innervated sections, indicating that the

**Figure 5. Nociceptive assay for annulus erector muscle function following ectopic and eutopic transplants.** A ventral view of several segments is shown. **a)** Relaxed juvenile leech that received an ectopic transplant of dorsal body wall at approximately 50% ED. **b)** Same leech immediately after immersion in 8% EtOH in leech saline. Although there was some local scarring, which produced irregularity, contraction of the body wall and erection of the annuli indicated that muscles in the segment that received the transplant (boundary indicated by black brackets) had become functionally innervated. **c)** Erect annuli in a juvenile leech that received a eutopic transplant of ventral body wall at approximately 50% ED. **d)** Exposure to EtOH caused annulus erection in 100% of embryos following either ectopic and eutopic transplants.

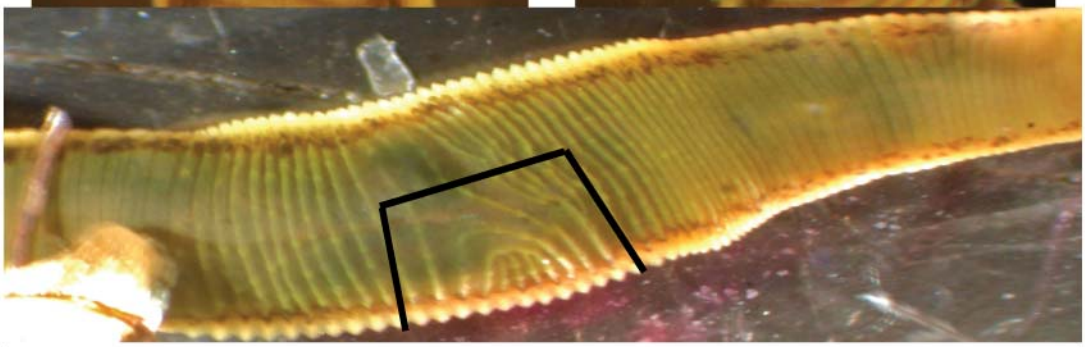
a



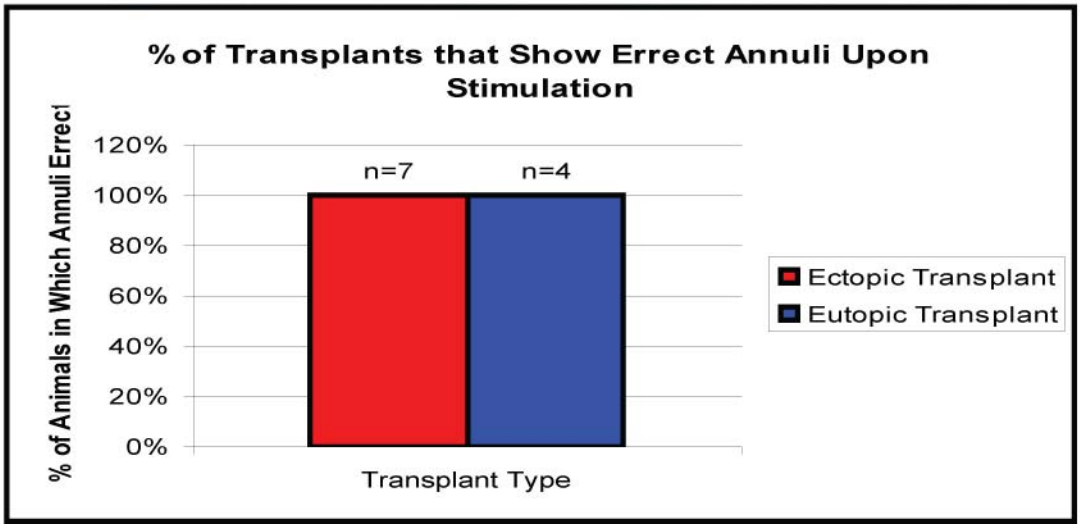
b



c



d


% of Transplants that Show Errect Annuli Upon Stimulation
% of Animals in Which Annuli Errect
120%
100%
80%
60%
40%
20%
0%
n=7
n=4
Transplant Type
Ectopic Transplant
Eutopic Transplant

response depends on neuronal input to these muscles and is not a response of the muscle fibers to the EtOH.

*Local innervation of the transplanted body wall*

Annulus erection in the patch of ectopic transplanted tissue suggested--but could not confirm--that the muscles in the transplanted tissue were functionally innervated, and it could not reveal whether the observed innervation was from the ganglion in that segment or whether axons from neighboring segments had (abnormally) grown into the transplant. To determine whether motor neurons in the segment had extended into the transplant, we filled identified motor neurons with Alexa flour 488 (green) or Alexa flour 568(red) and imaged the ganglion. We focused on two motor neurons that have been very well characterized: the dorsal longitudinal excitatory motor neuron DE-3 (cell DE-3--which, like most leech motor neurons, projects out of the ganglion contralateral to its soma) and the ventral longitudinal excitatory motor neuron VE-4 (cell VE-4--which is unusual in that it projects out of the ganglion ipsilateral to its soma). We found that fixation and further tissue processing reduced the intensity of the fluorescence and made it impossible to visualize axons outside of the ganglia, so in these experiments we imaged the filled cells in living body wall, which gave a stronger signal, even though the tissue could not be cleared.

In almost all cases the motoneurons projected out from the ganglia and into the adjacent segment (Fig. 6a,c,d). However, when cell DE-3 was filled in the eutopic transplants, its axons remained within the CNS and projected anteriorly in the connective (Fig. 6b,d). ( See Discussion)

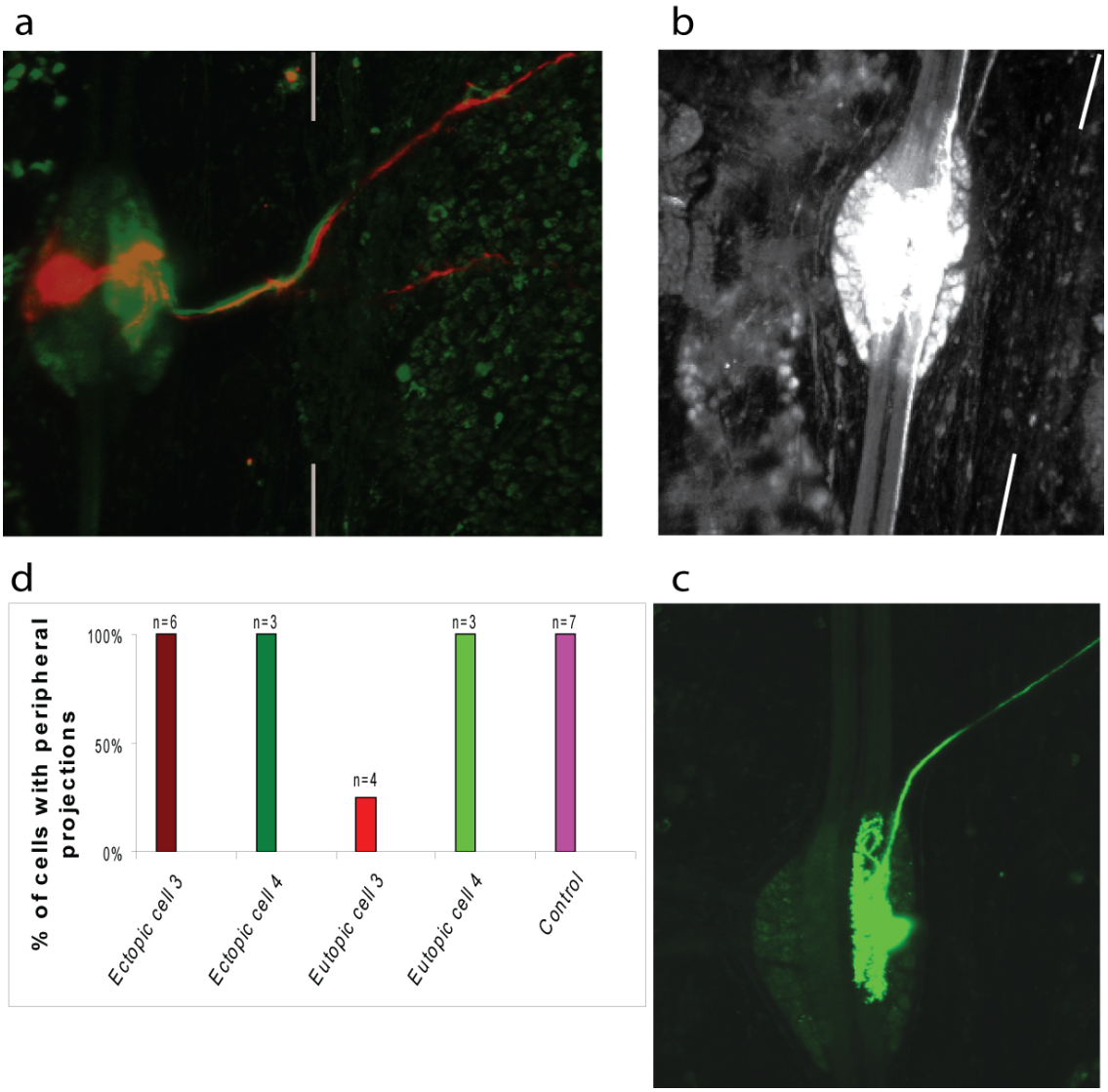



**Figure 6. Projection of individual motor neurons into the body wall revealed by filling single identified neurons.** Normally cell DE-3 projects out of the ganglion contralateral to the soma, whereas cell VE-4 projects out of the ganglion ipsilateral to the soma. **a)** Projection into a region where dorsal tissue was ectopically transplanted into a ventral position. The dorsal longitudinal exciter motor neuron cell DE-3 was filled with a red fluorescent dye; the ventral longitudinal exciter motor neuron cell VE-4 was filled with a green dye. Both motor neurons, cell DE-3 (red) and cell VE-4 (green), entered the transplanted tissue. (White tick marks signify the approximate medial boundary of transplants. **b)** Cell DE-3 following eutopic transplant of ventral tissue into a ventral location. No axon entered the periphery. **c)** Cell VE-4 following eutopic transplant of ventral tissue into a ventral location. The axon of this neuron exited the ganglion and entered the transplanted ventral tissue. **d)** Summary of peripheral innervation patterns.

*Dye-coupling of neurons following embryonic transplants.*

Previous work, in which the primordial reproductive duct tissue was ablated, showed that when the peripheral axons of embryonic leech Retzius neurons were not allowed to reach their peripheral targets the central synaptic connections made by these neurons changed. (Loer, et al., 1987) We wished to determine if changing the identity of their peripheral target could change central connectivity of the motor neurons. We hypothesized that forcing motor neurons that normally innervate ventral muscles to contact dorsal muscles might shift connectivity among the motor neurons.

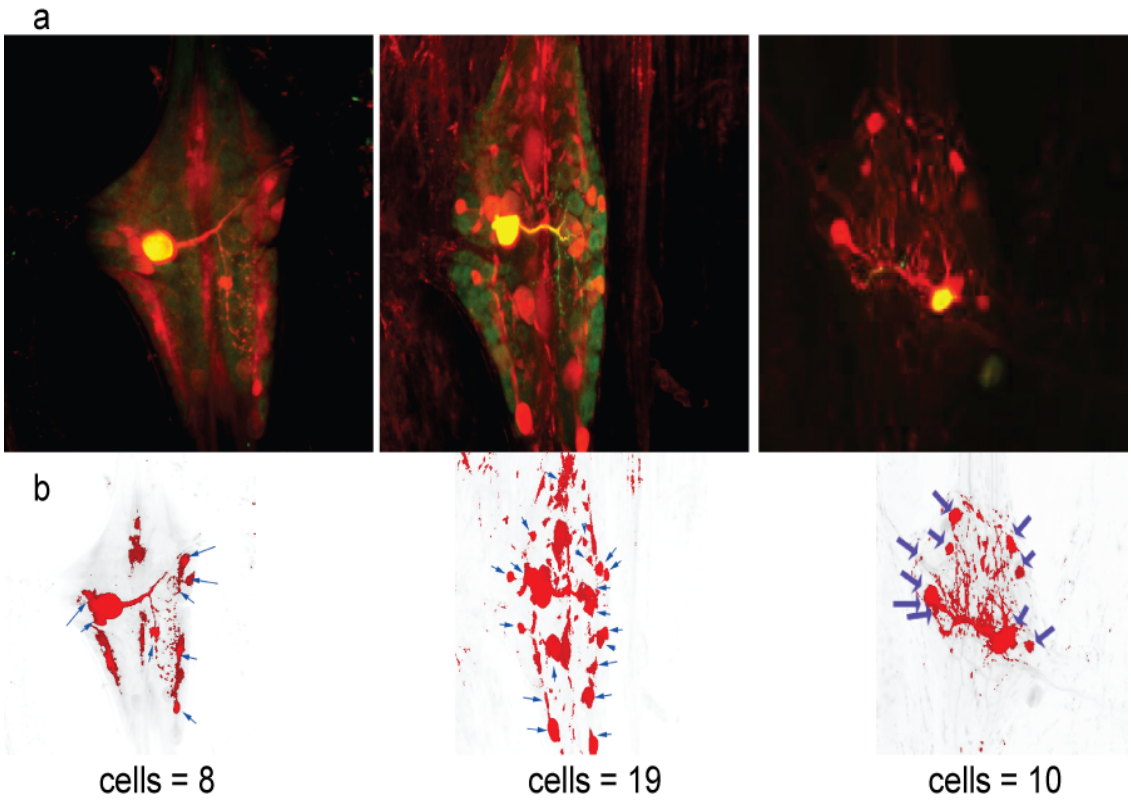
Unlike vertebrates, many invertebrates have both excitatory and inhibitory motor neurons. That is, some motor neurons directly excite muscles, but others directly inhibit them, and in the leech central nervous system, inhibitory motor neurons not only inhibit muscles, but they also make inhibitory synapses onto the excitatory motor neurons with which they share a target. We hypothesized that contact with muscles in the dorsal body wall might force an otherwise ventral motor neuron to form connections typical of a dorsal motor neuron. For example, might forcing cell VE-4 to contact dorsal tissue change the number or identity of neurons with which it forms a synaptic partnership within the ganglion?

Previous work (Marin-Burgin, et al., 2006) revealed that synaptogenesis in leeches passes through two phases: initially cells become dye-coupled, which can be assayed using the small molecule Neurobiotin, and later they may form chemical synapses. To ask whether forcing identified motor neurons to contact abnormal targets changes central connections, we began by assaying dye-coupling among neurons in

experimental embryos at a stage when previous results suggested that cells would be coupled in predictable patterns with other neurons.

We found that following transplants, individual motor neurons were dye-coupled to other neurons in the ganglia (Fig. 7a,b), but the number of coupled cells was highly variable among embryos, and we found no statistically significant differences among our groups (Fig. 7c). We have not yet been able to determine whether transplanting peripheral tissues alters the identity of the neurons to which the identified motor neurons are coupled.

**Figure 7. Dye-coupling of neurons following embryonic transplants.**
**a)** Dye coupling in three juvenile leeches visualized using Neurobiotin injection. All three leeches were treated as described in 'methods'
**b)** High-contrast images of the ganglia shown in panel a. **c)** Number of cells dye-coupled to cells DE-3 and VE-4 at the juvenile stage; 100% of development.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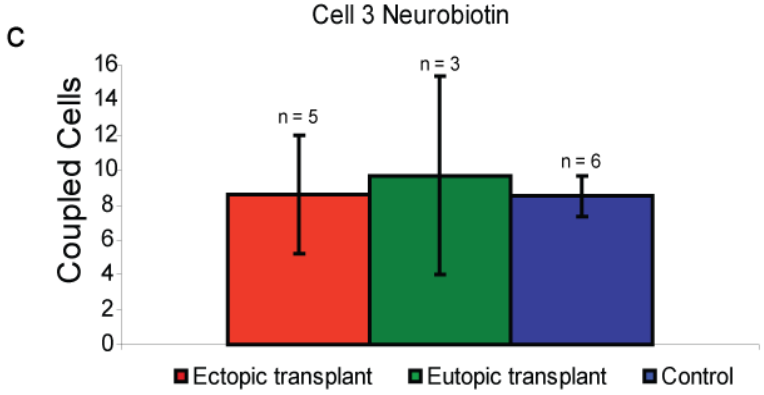
c
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Coupled Cells
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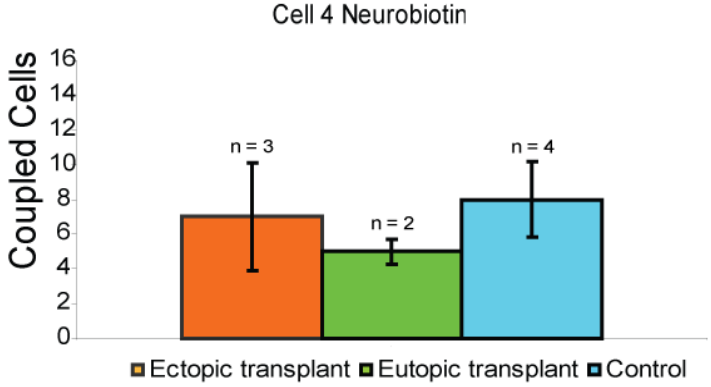
14
12
10
8
6
4
2
0
n = 5
n = 3
n = 6
Ectopic transplant
Eutopic transplant
Control


Cell 4 Neurobiotin
Coupled Cells
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14
12
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8
6
4
2
0
n = 3
n = 2
n = 4
Ectopic transplant
Eutopic transplant
Control

## DISCUSSION

### *Transplanted tissue becomes innervated*

Using antibody staining for ACT to reveal peripheral innervation, we found that transplanted tissue became innervated when the tissue was transplanted at ≈ 48% ED, whether it was moved to a novel location (ectopic transplants) or to its normal location (eutopic transplants).

### *Peripheral growth following transplantation*

We found that after ectopic transplants, axons of both cell DE-3 and cell VE-4 grew into the transplanted area, but following eutopic transplants only cell VE-4 sent axons into the transplanted area, while cell DE-3 projected only up the connective.

The behavior of cell DE-3 following eutopic transplants is consistent with previously reported results (Johnson, et al., 2000). In those experiments, the nerve roots on one side of a single ganglion were cut in embryos at about 50% ED, isolating one side of the ganglion from the periphery. Several days later, identified motor neurons were filled to determine their morphology. No peripheral tissue was transplanted, but these root-cut experiments share features with our eutopic transplants, because in both types of experiment, axons of cell DE-3 were severed (along with many other axons), and if they sprouted in the severed roots, the first tissue they encountered was ventral body wall. In both types of experiment, cell DE-3 sent axons up the connective, rather than into the periphery. Johnson, et al. (2000) also reported that other motoneurons, in their case the ventral inhibitory motor neuron VI-2, projected normally into the periphery despite the

root cuts. Previous work has shown that many neurons find their peripheral target by following cellular cues and gradients of adhesivity, while others--the pressure-sensitive mechanosensory neurons (P cells) that innervate the dorsal body wall area of interest here--send out a number of large and complex growth cones, one of which will find its target and form the peripheral axon while the others are eliminated.(Kuwada 1985). This P cell axon pioneers the pathway to the dorsal body wall, and many other axons that project to the dorsal body wall fasiculate with and follow that axon. The response of cell DE-3 in both types of experiments suggests that following the surgery this motor neuron has lost its normally pioneered pathway and is unable to respond to any remaining peripheral cues. However, if dorsal tissue is located immediately outside the ganglion, as it is following ectopic transplants, the growth cone of cell DE-3 recognizes the features of its normal peripheral target and readily grows into the tissue.

In contrast to cell DE-3, cell VE-4 projected into the periphery 100% of the time whether the transplanted tissue was removed from the dorsal or ventral body wall (Fig. 6 d). Perhaps the growing axons of neurons that innervate ventral tissues (e.g, axons of cell VE-4) have a simpler guidance program because their target tissue is normally located right outside the ganglion.

*Origin of innervation*

We wished to identify at least some of the neurons that were revealed by ACT immunocytochemistry. Of most interest was cell VE-4 which, after ectopic transplants, was left with none of its usual target tissue in its own segment. We found that cell VE-4

in the ganglia of transplanted segments projected out into the periphery, but did it then synapse onto tissue in the physical location typical to these motoneurons? Or, did it then follow signals that drove it into the ventral region of neighboring ventral segments (when normally a motor neuron innervates muscle fibers in only its own segment)? In previous work Wigston and Kennedy (1987) studied the hierarchy of reinnervation in the axolotl, asking whether it was a positional cue or an identity cue that guided the reinnervation of transplanted tissue. They injected a retrograde axonal tracer into a target muscle (ILT muscles) and then removed both anterior and posterior ILT muscles from the injected limb and replaced these muscles with their counterparts from the opposite limb, in reversed anterior/posterior orientation. After this transplant had healed, a second retrograde tracer was injected into the target muscle to determine if the muscles had been reinnervated by the same neurons as originally (which would suggest a re-innervation based on identity rather then position) or by a different neuron (suggesting re-innervation based on position). In these experiments, the majority of neurons selectively reinnervated muscles based on the identity/fate of their target rather then on the position of their target. Conversely, Smith and Frank (1987), working in the bullfrog, transplanted tadpole thoracic dorsal root ganglia (containing the somata of sensory neurons) to a brachial level to determine if the sensory neurons would innervate muscles appropriate to their novel location and if these neurons would then form central connections characteristic of their new location. In these experiments, neurons from the transplanted ganglia project appropriately for their novel location (rather then for their original identity).

These conflicting findings make it hard to predict the details of peripheral motor neuron function following transplants. Future physiological assays at the level of single identified motor neurons will address these issues directly.

*Did peripheral contacts alter central connections?*

The short answer is "Not that we could tell," However, although the number of cells dye-coupled to single identified motor neurons did not change following transplants, a great deal about the motor neurons did change. It was often hard to identify cells VE-4. In normal ganglia, there is a relatively stereotypical and well-characterized layout of the cell bodies. Thus, knowing which cell to fill is a matter of looking at the ganglion and comparing it to a map (Muller K. et.al., 1981) and then, once the cell is filled, confirming the cell's identity by observing the pattern of its projection within the ganglion and into the roots. In the ganglia of many segments that received transplants, the central morphology was atypical. Cell bodies were often not in a typical location, or they were smaller or larger then normal, and often when a cell was located in a typical position and had the expected soma size, it would not project normally (sometimes even to the point where it would project out of the "wrong" side). In these cases, it was impossible to determine from anatomy whether the soma position was "right" and the projection was "wrong," or whether the filled neuron was occupying an abnormal location. We neglected the data from these ambiguous situations because we lacked an independent indicator of cell identity. The burning question remains: did we discard data from the ganglia that most directly addressed our initial question? Did we ignore the ganglia that were most

affected by the transplants? The answers to these questions await further experimental study.

In closing, we found that, following ectopic transplants in the embryonic leech, transplanted tissue retained its original fate, was innervated both visually and functionally, and received innervation from the expected segmental ganglia. We also saw that forcing motor neurons to innervate these 'wrong' targets did not change the number of neurons electrically coupled to the motor neurons of interest, but did alter the size, location, and/or projection patterns of these motor neurons.

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