Title
Formation of appropriate synapses in the nervous system of the medicinal leech

Permalink
https://escholarship.org/uc/item/7zb139hb

Author
Todd, Krista L.

Publication Date
2009

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA, SAN DIEGO

Formation of appropriate synapses in the nervous system of the

medicinal leech

A dissertation submitted in partial satisfaction of the requirements for
the degree of Doctor of Philosophy

in

Biology

by

Krista L. Todd

Committee in charge:

       Professor William B. Kristan, Jr., Chair
       Professor Marla Feller
       Professor Kathleen French
       Professor Yishi Jin
       Professor Maryann Martone
       Professor Allen Ryan
       Professor Nicholas Spitzer

2009

The Dissertation of Krista L. Todd Is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

_____

_____

_____

_____

_____

_____
Chair

University of California, San Diego

2009

DEDICATION

To Anna, for seeing me through the many challenges along the way

TABLE OF CONTENTS

LIST OF FIGURES

Chapter 2

Chapter 3

ACKNOWLEDGEMENTS

Prior to my coming to the University of California at San Diego, I was fortunate to benefit from the mentorship of a dedicated and caring teacher at the University of Northern Iowa: Dr. Darrell Wiens. Dr Wiens helped me to understand the role of scientific research and provided me the experience of doing experiments that actually had an impact on how we understand organismal development. My research in that lab allowed me to attend and present data at my first national scientific meeting. For that experience and for helping me to establish a firm foundation in experimental science, I am very lucky to have been able to work with Dr. Wiens as an undergraduate.

Upon my arrival at UCSD, I was again extremely fortunate to have the opportunity to work closely with two great advisors and scientists, Dr. Kathy French and Dr. William Kristan. Kathy and Bill are adept at tackling large scientific questions ranging from behavioral decision-making in adult animals to the development of nervous systems in embryos. Amidst this fantastic scientific background I was allowed the freedom to develop my own experimental techniques and to follow the questions it generated. Bill and Kathy have always been there to provide much needed scientific advice, as well as the

framework for developing logical and well-controlled experiments. I am grateful to have gotten to work with such fine people.

There have been a number of post-docs and graduate students whom I have had the pleasure of working alongside in the lab: Quentin Gaudry, Mike Baltzley, Daniel Wagenaar, Antonia Marin-Burgin, Mike Baca, Kevin Briggman, and Eric Thomson. These friends and scientists contributed to the collegial atmosphere in the lab and were always excellent sounding-boards for new ideas and sources of creative solutions for difficult problems. Over the years, I have been privileged to work with a number of very talented undergraduates and a Masters student whose hard work and enthusiasm made working in the lab fun. These students include Sandahl Nelson, Andrea Cornford, Jillian Sola, Madeleine Geisheker, Alex Banach, and Vaibhav Konanur. I am also grateful to our lab manager, Joyce Murphy, without whose skills at encouraging leeches to reproduce, none of my experiments would have been possible.

I would like to thank my thesis committee, Drs. Marla Feller, Nick Spitzer, Maryann Martone, Yishi Jin and Allen Ryan, for their input and constructive criticism at our annual meetings. I am fortunate to have had the opportunity to interact with such a crowd of acclaimed scientists. I would like to specifically thank Marla Feller for allowing me

to work together with her in a classroom setting. Nick, Marla, and Maryann all gave advice regarding specific experiments contained in this thesis, and I thank them for that.

Chapter 4 of this thesis contains work that was done in conjunction with Master's student Sandahl Nelson, and presented here with her permission. These data are currently being prepared in manuscript form.

VITA

March, 1973	Born, Washington D.C.
2002	B.A., Biology, University of Northern Iowa, Cedar Falls, Iowa
2003 – 2009	Graduate Student Fellow, Department of Biology, University of California, San Diego, La Jolla, California
2004 – 2008	Teaching Assistant, Departments of Biology and Neurosciences, University of California, San Diego, La Jolla, California
2009	Teaching Assistant, Woods Hole Marine Biological Lab, Woods Hole, Massachusetts
2009	Ph.D., Biology, University of California, San Diego, La Jolla, California

PUBLICATIONS

Zaidi F, Todd K, Enquist L, Whitehead MC (2008) Types of Taste Circuits Synaptically Linked to a Few Geniculate Ganglion Neurons. J. Comp. Neurol. December 20; 511(6): 753–772.

Todd K, Kristan WB Jr., French K (2009) Gap Junction expression is required for normal chemical synapse formation. Submitted.

FIELDS OF STUDY

Major Field: Neurobiology
Professors Kathleen French and William B. Kristan, Jr.

ABSTRACT OF THE DISSERTATION

Formation of appropriate synapses in the nervous system of the

medicinal leech

by

Krista L. Todd

Doctor of Philosophy in Biology

University of California, San Diego, 2009

Professor William B. Kristan, Jr., Chair

The ability of complex organisms to sense their environment,
make decisions, and behave appropriately relies upon the synaptic
connections within their nervous systems. Developmentally, neurons
reliably form predictable connections onto other neurons. My work
examined how neurons choose their specific synaptic partners. We
chose the leech to study the developmental formation of synapses
based on its simple nervous system, identified neurons, characterized
neuronal development, and known behavioral circuitry. Chapter 2
demonstrates that electrical synaptic connections are required prior

to chemical synaptogenesis to permit the development of appropriate chemical synapses between neurons. We used RNA interference in single embryonic neurons to transiently decrease the mRNA of innexin-1, a component of electrical synapses in the leech CNS. Pressure-mechanosensory (P) cells treated in this manner failed to form a normal chemical synaptic connection onto a known post-synaptic partner, the Anterior Pagoda (AP) cell. Each of the four P cells in a midbody ganglion transduces sensory information from a quadrant of the leech's body wall and through chemical synapses with 24 interneurons mediates an avoidance behavior called a local bend. Disrupting the expression of electrical synapses in a single P ablated the behavioral response to stimuli within that P cell's receptive field. Treated P cells transduced sensory input, but failed to transmit that information to postsynaptic neurons. Chapter 3 describes a novel technique to study motor neuron development in leech embryos. Somata of most leech motor neurons lie on the dorsal surface of each ganglion, facing the interior of the animal. Thus, following the development of motor neurons over time requires that embryos must be dissected, embedded in agarose, and maintained in organotypic culture. This technique allowed us to keep embryos healthy many days longer than previously reported, revealing to us that the RNA

interference method of the previous chapter was effective in motor

neurons, as well as in sensory neurons. Chapter 4, reports how

transplanting peripheral target tissues affected the morphology and

central synaptic connections of developing motor neurons. After

transplanting pieces of body wall either ectopically or eutopically, we

determined how the presence of this altered peripheral tissue

affected neuronal development.

Chapter1: Introduction

Synapses in the nervous system

The nervous system of a human is made up of hundreds of billions of neurons interconnected in precise networks that provide the basis of thought, decision-making, and behavior. The functional unit of neuronal connections is the synapse, a region of specialized proteins localized to the sites where two neurons come into proximity with one another. The many proteins in each synapse constitute the macromolecular structures that are the gate through which information flows in neural networks. Constructing these complex structures requires the proper functioning of a list of other complex developmental processes (cellular differentiation, neuronal migration, axonal and dendritic guidance, and partner selection), and the formation of neuronal synapses is a highly regulated event.

Many lines of research have revealed that both vertebrate and invertebrate nervous systems first establish appropriate synaptic connections based on molecular mechanisms that establish a coarse initial pattern of synaptic connections (Godfrey, et al., 1984; Kirsch, et al., 1993; Kirsch, et al., 1993) and then by activity-dependent

processes that gradually refine them (Holland and Brown, 1980; Hubel and Wiesel, 1964; Thompson, et al., 1979; Torborg, et al., 2005; Wiesel and Hubel, 1963; Wiesel and Hubel, 1963; Wiesel and Hubel, 1965).

Two types of synapses connect neurons: electrical and chemical. At chemical synapses information is passed between neurons carried by neurotransmitter molecules that are released by one neuron, diffuse across a synaptic cleft, and act as ligands, activating receptors on another neuron.   At chemical synapses, information flow is uni-directional and therefore we can speak of pre-synaptic and post-synaptic neurons to describe their relationship.

Electrical synapses are composed of protein complexes called gap junction plaques. They are aqueous channels that connect the cytoplasmic compartments of the two coupled cells and allow the passage of ions and small molecules between them. Electrical transmission through these synapses is very fast, and can be bi-directional (although rectifying electrical synapses are also known). Gap junctions in the vertebrates are composed of protein sub-units called "connexins," whereas the subunits that form gap junctions in invertebrates are called "innexins" (Phelan, et al., 1998; Phelan and Starich, 2001). Connexins and innexins show little or no sequence

homology (Levin, 2002), although the function and the molecular structure of the complexes they form are similar.

History: electrical versus chemical transmission

The history of synaptic theory begins as most arguments (with proponents vehemently settled into two camps) and ends, as usual, with the truth lying somewhere in-between. The two camps were represented by two of the founding-fathers of modern neuroscience, the Italian Camillo Golgi, and the Spaniard Ramon y Cajal.

Golgi, along with several other well-regarded scientists, adhered to the Reticular Theory of the nervous system. This theory held that all the cells of the nervous system were connected in a syncytium. Cajal, on the other hand, believed that the nervous system was not continuous, but was separated into discrete cellular units. Interestingly, Cajal drew this conclusion based on observations of neurons stained using a method that Golgi had devised. Neither Golgi nor Cajal could suggest a mechanism for how a neuronal signal could be transmitted through the body either through the syncytium or among the cellular units. After sharing the 1906 Nobel Prize for Medicine, Cajal and Golgi each presented Nobel lectures attacking the other's view of neural connectivity. At the time of his death in

1926, Golgi still supported the Reticular Theory and believed that the cytoplasm of all cells in the nervous system was joined.

However, a growing body of anatomical evidence was casting doubt on Golgi's interpretations, showing instead that the nervous system consists of individual cells. Charles Sherrington termed the important gap between these cells "synapses" (Clarke E, 1996), and the search began to determine whether synaptic transmission was chemical or bioelectric. The chemical nature of synapses began to be elucidated when it was found that when adrenaline was applied to smooth muscles of the ileum and colon, it had an effect similar to activation of the sympathetic nerves innervating that region (Elliott, 1905).

Experiments done by Otto Loewi in the early part of the 20th century were crucial in demonstrating that synaptic signal transduction across most synapses was accomplished by small chemical compounds, the neurotransmitters. Loewi's elegant experiments involved dissecting two beating frog hearts, one with the vagus nerve still innervating the tissue. When he stimulated the vagus nerve, the innervated heart began to beat more slowly, and when he took some of the solution bathing that heart and placed it on the non-innervated heart, it, too, began to beat more slowly. This experiment

was the first definitive proof of chemical release from nerve cells, and it set off a hunt for other neurotransmitter compounds and seemed to end the debate whether synaptic transmission was chemical or bioelectric.

Then, in 1957, David Potter and Edward Furshpan provided the first evidence of direct electrotonic transmission between neurons (Furshpan and Potter, 1957). They were studying the tail-flip response of crayfish and discovered that sub-threshold stimulation of presynaptic neurons could elicit substantial potential changes in the post-synaptic neuron. We now know that these electrotonic junctions are composed of gap junctions, and they have been discovered in a variety of locations in both invertebrate and vertebrate nervous systems.

Electrical synapses and development

Although much attention has been focused on the function and development of chemical synapses, there is a growing appreciation that electrical synapses also play important roles in neuronal circuits (Bennett, 2000; Bennett, 2000; Deans, et al., 2001; Galarreta and Hestrin, 1999; Gibson, et al., 1999; Long, et al., 2004) and in their development (Drapeau, et al., 2002; Roerig and Feller, 2000; Tresch

and Kiehn, 2002). In many systems, neurons form transient electrical synapses at about the time when chemical synapses are forming (Peinado, et al., 1993; Penn, et al., 1994), suggesting that transient electrical synapses may be necessary for the formation of chemical synapses (Fischbach, 1972; Szabo, et al., 2004) or that they may contribute to activity-dependent refinement of chemical synapses by coordinating activity among coupled neurons (Kandler and Katz, 1995). These proposed mechanisms could best be tested directly by following a single neuron from its first synaptic contacts through to its adult set of connections. Recent work on fish (Drapeau, et al., 2002) and rodents (Bonnot, et al., 2002) suggests that this approach may some day be possible in a vertebrate CNS, but invertebrate nervous systems--with their identified neurons (Marder and Calabrese, 1996; Muller, et al., 1981)--provide the means for such a cell-by-cell analysis now.

In many nervous systems, the connections linking neurons change during embryonic development, and these changes may even be reflected in the behaviors the embryos can produce as they progress developmentally (Hamburger, 1963; Kiehn and Tresch, 2002). For instance, electrical coupling has been extensively observed in early development of the mammalian cortex (Schwartz, et al., 1998;

Yuste, et al., 1992), the mammalian retina (Feller, et al., 1996), the amphibian neural tube (Gu and Spitzer, 1997), and in spinal motor neurons of the developing chick (Milner and Landmesser, 1999; O'Donovan and Chub, 1997) and rodent (Tresch and Kiehn, 2000). These early connections have been thought to be made by axons that project broadly, setting up diffuse synaptic circuits that are then stabilized or pruned in an activity-dependent manner (Katz and Shatz, 1996; Ruthazer, et al., 2003). Such a pattern has been observed in the formation of the retinocollicular map (Debski and Cline, 2002; Ruthazer, et al., 2003), at the neuromuscular junction (Lichtman and Colman, 2000), and in the organization of the mossy fiber projection in the cerebellum (Crespel, et al., 1976).   However, recent experiments have revealed considerable specificity throughout the development of intracortical circuits (Borrell and Callaway, 2002; Bureau, et al., 2004; Katz, 1991). In these locations, synaptic connections arise with anatomic specificity and there is no need to prune back initially exuberant overgrowth; transient electrical synapses could provide a mechanism guiding this specificity by allowing initial recognition between synaptic partners. Such a mechanism would be parsimonious, because it would prevent growing neurons from

investing energy and metabolically expensive materials into extensions that will soon be demolished.

The relative time between the development of electrical synapses and the first appearance of chemical synapses strongly suggests that the two may be causally linked, but the hypothesis that an electrical connection between two neurons is necessary for the subsequent formation of chemical synaptic connection between them proved difficult to test directly.   However, the experimental tractability of the developing leech nervous system permitted us to approach the hypothesis and test it directly, with results that can now inform experiments to be carried out in developing vertebrates. Chapters 2 and 3 address this hypothesis.

Peripheral targets influence neuronal identity

The ability of tissues to affect the developmental fate of neighboring cells is one of the most important discoveries in the field of developmental biology. From the very earliest studies by Hilde Mangold, Hans Spemann, and others, induction has been known to play a powerful role in determining the eventual identity of embryonic cells. Studies in both invertebrates and vertebrates have shown that peripheral cues play an important role in shaping the central nervous

system (Baptista, et al., 1990; Loer and Kristan, 1989; Oakley, et al., 1997; Smith and Frank, 1987; Wenner and Frank, 1995). In the leech, the complement of individual identified neurons in each of the midbody ganglia is nearly identical from ganglion to ganglion along the ventral nerve cord, but there are notable exceptions. The large paired serotonergic Retzius cells in most ganglia of the ventral nerve cord project into the muscles of the body wall. However, in the two segments containing most of the reproductive structures (that, is midbody segments 5 and 6), the Retzius cells project to innervate the male and female sex organs (Loer and Kristan, 1989), and they differ from standard Retzius cells in central features, as well. When the reproductive tissue is ablated sufficiently early in development to preclude direct contact between the axons of Retzius cells and the primordial mesenchyme that develops into the reproductive ducts, the Retzius cells in those ganglia take on the anatomical and physiological characteristics of every other Retzius cell. Although direct transplants of developing reproductive mesenchyme proved to be technically impossible, experiments to explore cell lineages during development in the leech *Thermyzon* revealed that Retzius cells fated by lineage to assume the standard phenotype took on the reproductive segment phenotype when they contacted ectopic

reproductive mesenchyme (Gleizer and Stent, 1993). It thus appeared that the phenotype assumed by individual Retzius cells was guided by some signal from the reproductive tissue. In Chapter 4 we have done experiments to further test the ability of target tissues to affect neuronal identity by transplanting specific regions of the body wall into the path through which identified motor neurons send their axons to force the axons to contact inappropriate peripheral target tissues.

Concluding Remarks

The work contained in this thesis addresses the question of how neurons achieve their individual identity and make the specific connections that will allow the organism to behave appropriately in its environment. I have focused on the early period of leech embryogenesis, during which neurons are first extending their processes and making initial contacts with other neurons. I developed a single-cell RNA interference method that allows us to transiently depress the function of electrical synapses in intact embryos at specific stages of development. By recording intracellularly from two identified neurons that are normally connected monosynaptically through a strong chemical synapse, I discovered that when these two

neurons cannot form electrical junctions during the period of chemical synaptogenesis the chemical synapse between them is diminished or gone, and it fails to recover, even weeks or months later. I used semi-intact preparations to determine that the RNAi treatment produced a behavioral deficit that persisted months after the treatment. I now believe that electrical synapses in developing nervous systems are necessary to produce normal neuronal circuitry in the leech nervous system and that this requirement is likely to be universal among animals that depend on nervous systems to produce and control behavior.

Chapter 2: Gap junction expression is required for normal chemical synapse formation

Summary

Electrical and chemical synapses provide two distinct modes of direct communication between neurons, and the embryonic development of the two is not simultaneous. Instead, gap junction-based electrical synapses arise prior to chemical synaptogenesis, and where it has been studied, the anatomical connections and the activity patterns they produce resemble the mature, chemically-mediated circuits that appear later. The temporal correlations between electrical and chemical synapses led to the hypothesis that formation of electrical synapses is a necessary forerunner to chemical synaptogenesis, but that hypothesis has proven difficult to test directly. We used RNA interference in individual neurons to transiently reduce the expression of gap junction proteins in individual identified neurons during chemical synaptogenesis in intact leech embryos (*Hirudo verbana*). We then determined whether these neurons formed normal chemical synaptic connections. We found that reducing

gap junction expression was followed by failure to form expected chemical synapses, and this lack of chemical synapses persisted for several weeks after the initial treatment, even though normal electrical connections recovered within days. We conclude that electrical synapses provide cues critical for early chemical synaptogenesis, and their absence during the normal period of synaptogenesis permanently inhibited the formation of chemical synapses. Thus, connection via gap junctions is necessary to allow these neurons to form appropriate chemical synapses onto a particular synaptic target and, more broadly, to be integrated into a defined behavioral circuit.

Introduction

Electrical synapses are sites of neuronal communication where gap junction protein complexes allow both ions and relatively small biomolecules to pass between cells. Although electrical synapses were first characterized almost fifty years ago (Bennett, et al., 1963; Furshpan and Potter, 1959) most synaptic research has focused on chemical synapses. There is, however, a growing appreciation that electrical synapses are important in mature neuronal circuits (Drapeau, et al., 1995; Roerig and Feller, 2000) as well as in neuronal

developmental processes such as neuronal differentiation (Bani-Yaghoub, et al., 1999; Bani-Yaghoub, et al., 1999), radial migration (Elias, et al., 2007), and apoptosis (de Rivero Vaccari, et al., 2007).
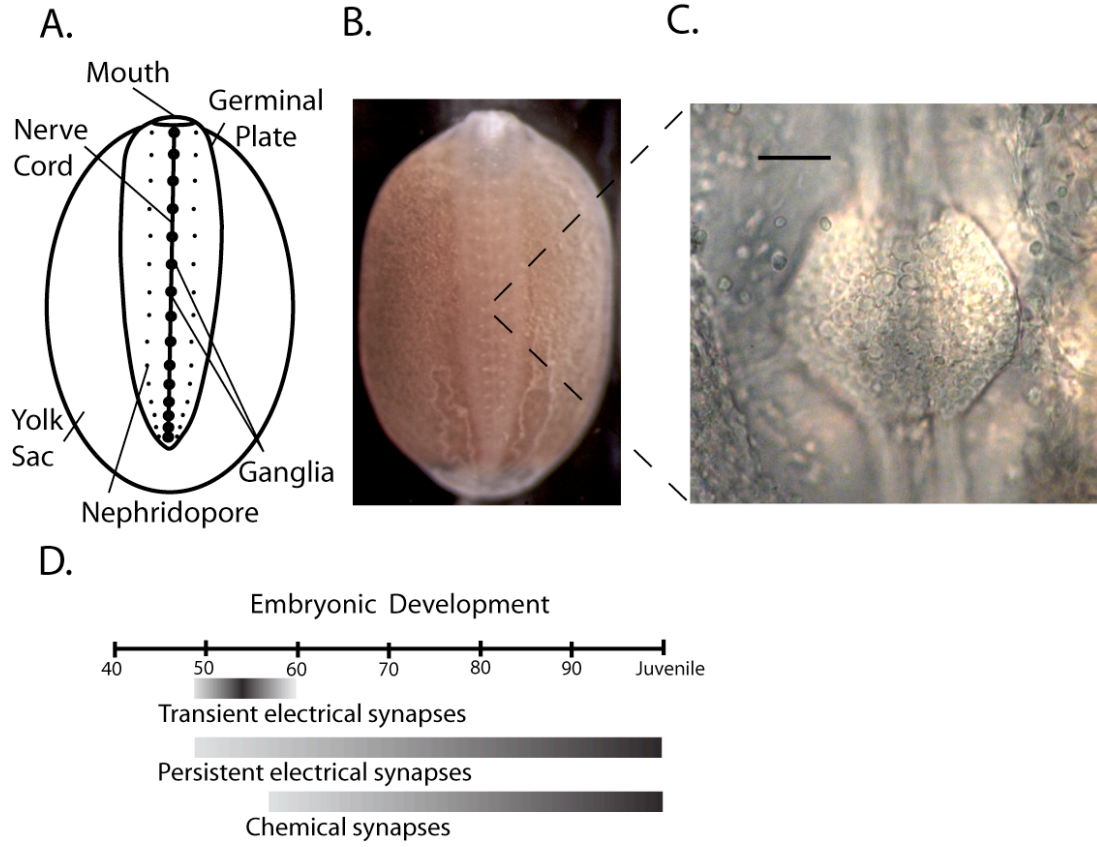
Electrical synapses may also contribute to the formation of neural circuits. It has widely been observed in many species (e.g., the optic lamina of *Daphnia* (Lopresti, et al., 1974), rat cortex (Peinado, et al., 1993), ferret retina (Penn, et al., 1994)) that neurons form transient electrical synapses just prior to the appearance of chemical synapses. Similarly, axons regenerating in culture form electrical synapses before establishing appropriate chemical synapses (O'Lague, et al., 1978; Szabo, et al., 2004). In *Drosophila*, mutations in either of two genes led to impaired electrical and chemical synapses in the visual system, and transgenic expression of electrical synapses during development rescued chemical synaptic transmission in adults (Curtin, et al., 2002). These correlations strongly suggest that transient electrical synapses may be necessary either in the formation of chemical synapses (Fischbach, 1972; Szabo, et al., 2004) or in their activity-dependent refinement (Kandler and Katz, 1995), but the difficulty in modifying the development of individual identified neurons *in vivo* has been an obstacle to a direct and specific test of either of these developmental programs.

However, in embryos of the medicinal leech, *Hirudo verbana* (previously not distinguished from *Hirudo medicinalis* (Siddall, et al., 2007)) we can follow an individual identified neuron's development from its initial neurite outgrowth to its incorporation into a mature functional neural circuit. We used these robust and tractable embryos to investigate the necessity of gap junctions in the process of chemical synaptogenesis. Even as embryos (Fig. 2.1A,B), the somata of the neurons in *H. verbana* ganglia are large (10 – 15 μm in diameter) (Marin-Burgin, et al., 2005), they are in stereotyped positions (Dietzel and Gottmann, 1988; Marin-Burgin, et al., 2005), and the ganglia are easily accessible through the body wall (Fig. 2.1C). In addition, the behavioral function of many leech neurons is known (Kristan, et al., 2005), as is the developmental timeline of neural connectivity (Marin-Burgin, et al., 2005) (Fig. 2.1D).

At least 12 different innexins (i.e., invertebrate gap-junction protein monomers) have been identified in *Hirudo* sp. (Dykes and Macagno, 2006), but *Hm-inx1* is the only molecule that is expressed in all central neurons. To test whether gap-junction based electrical connections are a necessary part of chemical synapse formation, we developed an *in vivo*, single-cell RNAi protocol that transiently decreases the functional expression of *Hm-inx1* (Dykes, et al., 2004)

Figure 2.1 *Hirudo verbana* **anatomy and timeline of synaptic development**

A. Anatomy of a leech embryo at about 50% of embryonic development (50% ED), the stage at which we injected siRNA into neurons. The germinal plate develops on top of a yolk-filled sac; the embryo grows toward the posterior and expands left and right until it encircles the yolk sac. B. Leech embryo at ~50% ED, the stage depicted in panel A. The nerve cord is visible on the midline through the transparent body wall of the animal. This embryo is about 6 mm long. C. The ventral surface of a segmental ganglion, viewed through a small incision in the body wall. Somata of about 200 neurons are visible in this ganglion. Scale bar is 50 μm. D. Timeline for development of electrical and chemical synapses in *Hirudo* (modified from Marin-Burgin et.al. 2005 (Marin-Burgin, et al., 2005)). Neurons lack processes before about 40 % ED, and the leech is becomes a juvenile at 100 % ED. 1 day of developmental time equals about 3% ED at room temperature (Reynolds, et al., 1998).

A.

Mouth

Germinal
Plate

Nerve
Cord

Yolk
Sac

Ganglia

Nephridopore

B.

C.

D.

Embryonic Development

| 40 | 50 | 60 | 70 | 80 | 90 | Juvenile |

Transient electrical synapses

Persistent electrical synapses

Chemical synapses

early in the period of synaptogenesis and observed the results of the

manipulation at the molecular, cellular, synaptic, and behavioral

levels.

Methods

Animals

Adult medicinal leeches (*Hirudo sp.*) 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 days after egg deposition and were then held at 20–

24°C in "embryo water," sterile-filtered Arrowhead spring water

(Arrowhead Water, Brea, CA) with 32 mol of $MgCl_2$ and 40 mol of

$CaCl_2$ added per liter.

Staging

We determined the embryonic stage of each embryo using

external morphological features that run from 0% (egg deposition) to

100% of ED (Reynolds, et al., 1998). Development proceeds at ~3% ED

per day at 20°C. All experiments were performed in ganglia from the

mid-body of the animal (segments 7–15) to minimize the effects of the

anterior–posterior gradient of development in the leech; ganglia in this

region differ by no more than 1% ED (Reynolds, et al., 1998).

Generating siRNA

We identified potential siRNA targets within the *Hm*-inx1 gene

using an online algorithm provided by Ambion

(http://www.ambion.com/techlib/misc/silencer_siRNA_template.html).

Multiple, 21-base sequences were chosen and were individually

generated by *in vitro* transcription with a Silencer siRNA Construction

Kit (Ambion). We used a mix of two sequences targeting the *Hm*-inx1

mRNA (5'- AATAACTACAGCATGGAGAGG -3', and 5'-

AAGAGATCCGGAGTCAATATG -3'). A scrambled-control sequence

(equal number of nucleotides to the first in a random order, non-

homologous to known genes) was also made (5'-

AATCGAGAAGAGCTAAACTGG -3'). The concentrations of the

resulting dsRNA were measured with a spectrophotometer and the

samples were frozen at -20º C.

Neuronal Injections

---Adult

Single midbody ganglia from adult leeches were dissected in

normal leech saline (Muller, et al., 1981). We pinned the ganglia in dishes coated with Sylgard® to access either the dorsal or ventral neurons. The injections were performed on an Axioskop 2 (Zeiss) equipped with both transmitted light and fluorescence optics. siRNA fragments were diluted to 10 – 15 µM in 1.5% AlexaFluor Dextran (10k mw, Invitrogen) in sterile water and then wicked into sharp microelectrodes that had resistances of 60 – 80 MΩ if filled with 3M KAc. Target cells were identified and impaled under transmitted light. The siRNA / Alexa mixture was pressure injected (100 psi) at ~ 1 Hz using a Picospritzer II (General Valve Corporation) and monitored using fluorescence optics. Since it was difficult to determine the volume injected into target neurons, fluorescence images were obtained through the filling process using an AxioCam Hrc (Zeiss) and AxioVision software (Zeiss) set at standardized acquisition settings. Once the average somatic pixel intensity was greater than 80% maximum value, the injection was stopped. These ganglia could then be maintained in sterile L-15 culture medium until needed for assays. The same method was used for injections of scrambled control RNA, and Alexa Dextran vehicle controls.

---Intact Embryo, Ventral Neurons

We selected embryos aged 50% or 60% ED from our breeding colony. They were anesthetized in 8% ethanol in normal leech saline and immobilized, ventral side up, in a Sylgard® trough using two latex straps. The nerve cord and ganglia were visualized with a dissection microscope. We then used a sharpened tungsten pin to make a small hole in the body wall above a target ganglion. The preparation was moved to a compound microscope and the injections were performed as above, using 80 – 100 MΩ electrodes. The embryos were then moved to a new 60 mm Petri dish containing 50% normal leech saline and 50% embryo water where they healed and continued developing.

Determining Number of Dye Coupled Neurons

Identified neurons were impaled, filled, and processed according to Marin-Burgin et. al, 2005 (Marin-Burgin, et al., 2005). Standardized acquisition settings were used to obtain confocal Z-stack images of the preparations (Bio-Rad). The stacks were then processed using ImageJ (http://rsbweb.nih.gov/ij/) to determine the ganglion's average pixel intensity and the standard deviation. A Look-Up-Table (LUT) was created that displayed pixels with intensities over 1 SD above

the mean. We could then look through the z-stack and use the 1 SD threshold to count coupled neurons.

In situ Hybridization

To reveal *Hm*-inx1 mRNA, we used an *in situ* hybridization protocol as described in Dykes et. al., 2004 (Dykes, et al., 2004).

Electrophysiology

--Adults

Adult ganglia were transferred from L-15 culture medium to cold, normal leech saline (Muller, et al., 1981). We recorded on the stage of a compound microscope equipped with both transmitted light and fluorescence optics using sharp microelectrodes with resistances of 40–60 M when filled with 3 M potassium acetate. Intracellular signals were recorded with an Axoclamp-2A amplifier (Axon Instruments, Foster City, CA) operating in current-clamp mode. We stimulated and acquired data using a PC-based system using pClamp 9.0 software (Molecular Devices Corporation, Sunnyvale, CA).

---Embryos

We obtained electrophysiological recordings from embryonic

neurons in a similar manner; only the differences will be noted. Under a dissection microscope, we opened the embryos with a dorsal incision and loosely pinned the body wall open to expose the dorsal surface of the ventral nerve cord. The embryo was moved to the compound scope and examined under fluorescence optics. This was done to verify that the correct target neuron had been treated and to mark which midbody ganglia would be used. We then re-pinned the embryo ventral-side up, noting which ganglion had been treated. The body anterior and posterior to the target ganglion was removed, leaving a patch covering 6 segments in the middle. An incision was made over the target ganglion and a control ganglion 2 segments away. The blood sinus (stocking) was removed over these ganglia with a sharpened tungsten pin and the body wall was pinned tightly all around. To further prevent motion artifacts, incisions were made at regular intervals in the body wall to sever longitudinal and oblique muscles. We recorded from neuronal somata using sharp microelectrodes that had resistances of 50–80 M when filled with 3 M potassium acetate. Embryonic neurons proved to be extremely sensitive to osmotic changes, so we filled our electrodes with a solution of 120mM potassium gluconate, which is nearly iso-osmotic with the external medium; their resistance was 200–600 M. To prevent photo-

toxic damage, cells were illuminated only very briefly during recordings.

---Chemical EPSP amplitude

We measured the EPSP amplitude for chemical synapses by passing a current pulse into the presynaptic neuron (P cell) while recording from the postsynaptic neuron (AP cell). Because the AP neuron fires tonically at its resting membrane potential, EPSPs were more easily measured by silencing the AP with a 0.5 nA hyperpolarizing current injection. This reduced the membrane potential to -60 to -70 mV. EPSP amplitude in the AP neuron is dependent on its resting membrane potential due to an inward rectifying K^+ current (Wessel, et al., 1999). Over this range, however, the effect is less than 0.5 mV.

---Electrical coupling strength

We measured the strength of an electrical synapse by passing multiple different current steps into one cell and recording the voltage responses in the coupled cell. These steps were passed alternately into both cells of the pair. To compute the coupling strength, we divided the voltage response in the coupled cell by the stimulus

current using a custom program written in MATLAB; the slope of this line is the coupling strength. This quantity is often called the "transfer" or "mutual resistance" (Bennett, 1966) but we prefer to use the more heuristic term "coupling strength" and report our results in units of millivolt postsynaptic per nanoampere presynaptic rather than in megaohms.

Behavioral Assay

---Video recording

Local bend behavior was elicited and video recorded according to Baca et al., 2008 (Baca, et al., 2008). Briefly, we recorded the image of the body wall preparations through a Wild dissection microscope using a C-Mounted Hitachi KP-M1 monochrome CCD camera (Image Labs International, Bozeman, MT). The images were captured at 10 Hz for 15 seconds using a Data Translation frame grabber card (DT3155) controlled with the MATLAB (The Mathworks, Natick, MA) Image Acquisition Toolbox on a PC computer. On a different computer, pulses from Axograph 4.9 software (Axon Instruments, Union City, CA) synchronized video acquisition with the stimulus controller.

---Body wall stimulus

The leech was opened along a ventro-lateral incision made on the side opposite the ipsi-laterally projecting Pd cell of interest. Most of the body was discarded, except for 5 segments centered on the target ganglion. We then denervated the two anterior and posterior segments. In order to avoid the stimulating the N (nocioceptive receptor) cells in the segment being recorded, we pinned the body wall only in the denervated anterior and posterior ends. In these preparations, we stimulated the Pd and ipsilateral Pv receptive fields with five stimulus force strengths, repeated three times in random order, with three minutes between trials. The actual force applied was verified using a lab balance placed under the preparation.

---Contraction analysis

As previously described in Baca et al. (Baca, et al., 2005), we tracked the body wall motion by making optic flow estimates between successive image frames. We used the frame with the peak contraction during the local bend behavior to determine the maximal body wall excursion within the targeted receptive field. Longitudinal motion across the entire body wall within 3 annuli anterior to the

stimulus was then integrated. This yields a body wall bend profile that displays the integrated contraction data distributed continuously across the body wall. We then extracted the maximum and mean contraction within each quadrant of the leech.

Results

Injecting siRNA into a single adult neuron decreased the level of mRNA coding for *Hm*-inx1.

To test whether RNA interference (RNAi) could decrease cellular levels of innexin-1 mRNA, we used 21-base-pair long double-stranded RNA (dsRNA) synthesized from chosen sequences within the *Hm*-inx1 gene. These dsRNAs were injected into one of the pair of Retzius neurons in ganglia from the ventral nerve cord from adult leeches. Following injection, ganglia were held in organ culture for several days. Normally, the two Retzius neurons within a ganglion are very strongly electrically coupled to each other (Hagiwara and Morita, 1962), and this connection is echoed by the results of *in situ* hybridization using an *Hm*-inx1 probe in control ganglia (Fig. 2.2A,B). In contrast, 2 days after dsRNA was injected into one of the pair of Retzius neurons, the staining intensity of the treated Retzius neurons was less than 25% of the untreated Retzius neurons.

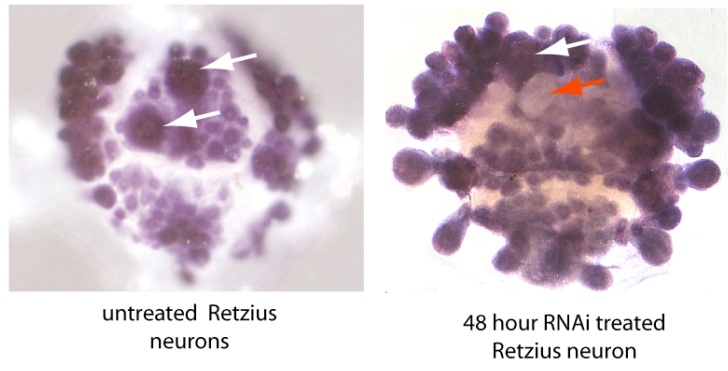**siRNA treatment disrupted electrical synapse function**

After injecting one of the paired Retzius neurons with siRNA or a scrambled RNA and holding the ganglion in organ culture for 1 - 5 days, we used sharp microelectrodes to simultaneously impale both Retzius neurons in the ganglion and applied a series of hyperpolarizing current steps to each neuron while recording the voltage deflection in the other. In untreated ganglia and in ganglia into which we injected a scrambled RNA, we recorded an average response in the follower cell of 5.4 +/- 0.6 and 5.2 +/- 0.8 mV per nA, respectively, of current injected into the driven cell, regardless of which cell of the pair was driven. The electrical synapse between Retzius neurons is non-rectifying (Muller, et al., 1981), i.e., current flows equally well in either direction. Following siRNA treatment, this signal decreased significantly, to 3.0 +/- 0.8 mV/nA at 24 and 2.9 +/- 0.2 at 48 hours after the treatment (Fig. 2.2C), regardless of which Retzius cell was driven (data not shown), and the coupling returned to normal values by 120 hours after treatment. Repeating these experiments in embryos at 60% ED reduced the electrical coupling between Retzius cells by more than 80% compared with untreated ganglia in the same embryos (Fig.

Figure 2.2 RNA interference decreased *Hm*-inx1 mRNA and coupling strength.
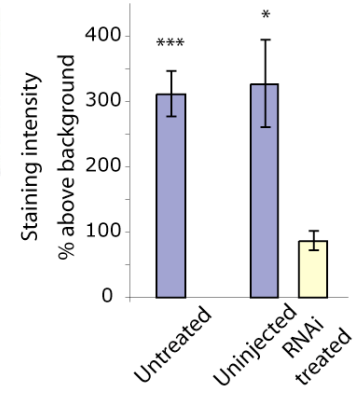
A. *In situ* hybridization using full-length *Hm*-inx1 probe produced dark staining (left) in untreated adult Retzius cells (white arrows), indicating high levels of *Hm*-inx1 mRNA. The mRNA level was lower 48 hours after siRNA was injected into only one of the Retzius cells (red arrow), while the second, uninjected neuron continued to stain darkly. B. Uninjected adult Retzius cells and those in untreated ganglia stained 8 times more darkly than Retzius cells injected with *Hm-inx1* siRNA 48 hours prior to staining (n = 8 untreated, 4 uninjected, and 4 RNAi treated. Error bars show SEM, * $p < 0.05$, *** $p < 0.001$). C. Coupling strength, a measure of the electrical conductance between the two Retzius neurons, was decreased at 24 and 48 hours. Coupling strength returned to normal values by 120 hours after the treatment. The cartoon illustrates this assay: blue circles represent the two adult Retzius cells in a normal ganglion. Five hyperpolarizing current steps (.5, 1, 1.5, 2, 2.5 nA) were passed into each Retzius cell and the voltage change elicited in the other cell was recorded. The coupling strength was the slope of the line fitted to a plot of voltage change in the second cell as a function of the amount of current passed into the first cell. In this figure and all subsequent figures, blue signifies no injection of siRNA; purple signifies injection of a scrambled, non-functional siRNA, and pale yellow signifies injection of siRNA targeting *Hm*-inx1 (24 hours, n = 10 uninjected, 6 scramble-injected, and 10 RNAi treated; 48 hours, n = 22 uninjected, 12 scramble-injected, and 20 RNAi treated; 120 hours, n = 7 uninjected, 7 scramble-injected, and 6 RNAi. Error bars show SEM, ** $p < 0.01$, *** $p < 0.001$)
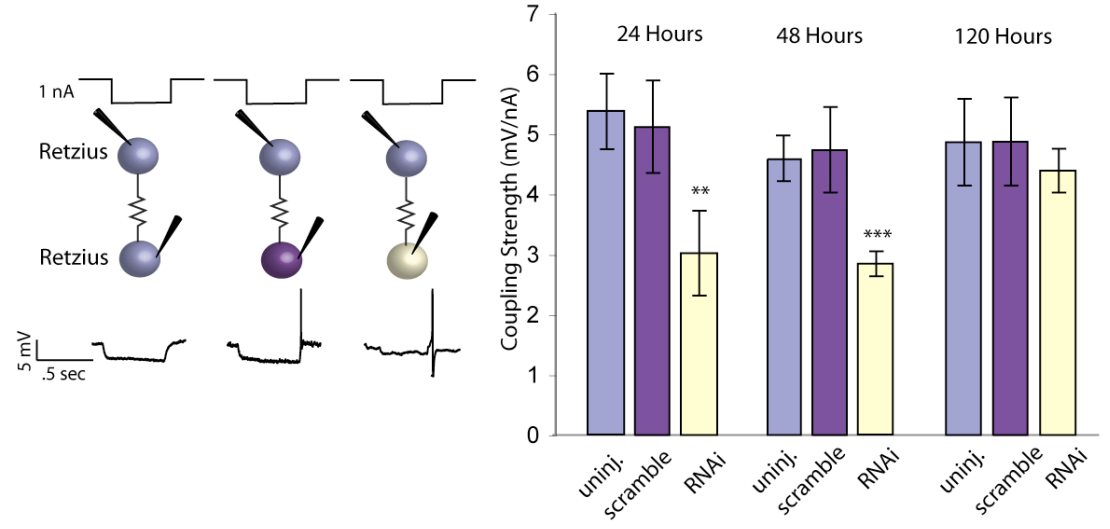
A.

untreated Retzius
neurons

48 hour RNAi treated
Retzius neuron

B.

C.

1 nA

Retzius

Retzius

5 mV
.5 sec

24 Hours   48 Hours   120 Hours

uninj. scramble RNAi   uninj. scramble RNAi   uninj. scramble RNAi

2.3A, B). We tested the efficacy of several siRNA sequences targeting

different portions of the *Hm-inx*1 mRNA and discovered that, as

expected, not all siRNAs performed equally well. Two sequences,

siRNA A (targeting the 5' region) and siRNA B (targeting the middle

region), both yielded a significant decrease in coupling strength

compared to uninjected and scrambled dsRNA-injected controls. In

the rest of our experiments we combined these two sequences in all

injections, which we will call inx1-siRNA.

A second measure of electrical synapse function is how readily

the gap junctions pass small molecules. To test the effect of RNAi on

other types of neurons in embryos, we injected inx1-siRNA into one

pressure-sensitive mechanosensory neuron (a P cell) in embryos at 65%

ED (Fig. 2.1D). We incubated the embryos for 48 hours, then filled the

treated cells with Neurobiotin, a small molecule that passes through

gap junctions between embryonic leech neurons (Fan, et al., 2005;

Marin-Burgin, et al., 2006). After processing with fluorescently labeled

streptavidin, we counted the number of neurons dye-coupled to the

treated neuron and found that the number of neurons dye-coupled to

the RNAi-treated neurons was significantly reduced from control

values (Fig. 2.3C). From the experiments presented so far, we

conclude that intracellular injection of inx1-siRNA reduced the

Figure 2.3 siRNA targeting *Hm*-inx1 decreased function in embryonic electrical synapses

A. Current and voltage traces of embryonic Retzius-to-Retzius electrical coupling. 1 nA of hyperpolarizing current was passed into one Retzius cell for 0.5 sec. and a voltage deflection was recorded in the other Retzius cell. Blue indicates an uninjected Retzius cell, purple a Retzius cell injected with scrambled siRNA, and pale yellow a Retzius injected with active siRNA. Injections were made at 60% ED, and the recordings were performed 48 hours later, at 66% ED. B. Many different siRNA sequences were tested. Sequences arbitrarily called "RNAi A and RNAi B" significantly decreased electrical coupling strength 48 hours after treatment. (ANOVA, * $p < 0.05$, *** $p < 0.001$. Significance is compared to both uninjected and scramble-injected) C. In each ganglion, a single mechanosensory P cell (red arrows) was injected with Neurobiotin 48 hours after it was treated with RNAi. The darkened cells are neurons dye-coupled to the Neurobiotin-injected P cell. The number of cells coupled to the P cell was lower in treated ganglia assayed 48 hours after treatment (ANOVA, $p < 0.001$).

A.

B.
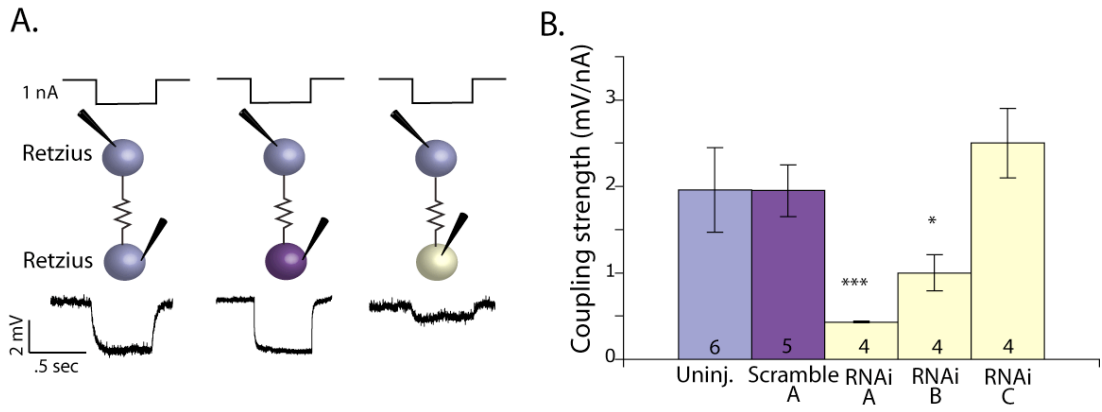
C. Pressure mechanoreceptor (P Cell)
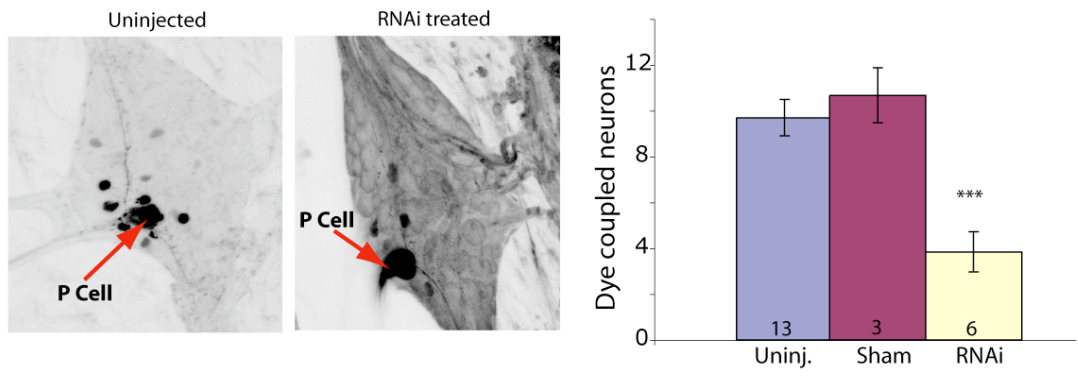
electrical synapses formed among leech neurons, as assayed by *in situ* hybridization, electrical coupling, and the ability to exchange the small molecule, Neurobiotin.

Weakening electrical synapses disrupted chemical synapse function

Previous work determined that *Hm-inx*1 mRNA is expressed in the nervous system beginning at about 48% ED (Dykes and Macagno, 2006), and electrical synapses can be detected electrophysiologically within a day, at ~51% ED (Marin-Burgin, et al., 2005). In contrast, there is no indication of chemical synapses in embryonic ganglia until at least two days later, at ~55% ED. We used mechanosensory P cells because of their pivotal role in eliciting local bending behavior (Thomson and Kristan, 2006). To directly test the influence of electrical synapses on chemical synaptogenesis, we used mechanosensory P cells because they elicit local bending (Thomson and Kristan, 2006), a well-studied behavioral circuit in the leech CNS (Kristan, et al., 2005). Sensory input into the circuit comes entirely from the four mechanosensory P cells in each ganglion; each P cell is responsible for transducing pressure sensory information from a quadrant of the body wall within the segment in which the ganglion is located (Lewis and Kristan, 1998; Nicholls and Baylor, 1968). When Neurobiotin is injected

into an adult P cell, 12 other neurons within the same ganglion are labeled, and one of them is the contralateral AP cell, a motor-like neuron of unknown function. In adult *Hirudo* (as well as in another leech species (Ren-Ji Zhang, 1995)) the two neurons are connected by a robust excitatory chemical synapse made by the P cell onto the AP (Wessel, et al., 1999) with a weak electrical connection, but we have found that early in development, the coupling between these two neurons is completely electrical (data not shown).

We injected inx1-siRNA into a single embryonic P cell at 48 – 50% ED, a time when innexin-based electrical synapses are initially forming, but chemical synapses are not yet present (Marin-Burgin, et al., 2005). After the injection, we allowed the embryos to heal and to continue developing normally. One week later, at 70% ED, we simultaneously impaled P and AP cells with sharp microelectrodes to evaluate chemical synapses between the treated P cells and their contralateral AP cells (Fig 2.4A). In control animals, the P cell had formed a strong chemical synapse onto the AP cell: action potentials in the P cell evoked robust EPSPs (7.1 +/- 1.1 mV) in AP cells that had been hyperpolarized to silence tonic firing. In contrast, following inx1-siRNA treatment, action potentials in a P cell elicited EPSPs in the AP cell that were typically less than 1 mV and were thus generally were obscured

by background noise (Fig 2.4A, B). In a second measure of the strength of the P-to-AP synapse, we stimulated the P cell to produce single spikes and determined the percentage of trials in which these spikes elicited time-locked spikes in AP cells was about 80% of the time. After RNAi, P cell spikes elicited a significantly lower percentage of AP action potentials (45% lower) when compared to uninjected control animals (Fig 2.4C).

We know from data presented in Fig. 2.2C that injecting dsRNA had a transient effect that lasted no more than 5 days. To determine whether injecting dsRNA into neurons at 48% ED merely delayed the onset of chemical synaptogenesis that would eventually produce chemical synapses after electrical connections were re-established, we injected dsRNA into P cells at 48% ED then tested the strength of their synaptic connections onto AP cells when the leeches became juveniles, from 21 to 35 days after the injection. Even after this long period, the amplitude of EPSPs in AP cells remained small (Fig. 2.4B), and the percentage of P cell spikes that elicited a spike in the AP cell was low (Fig. 2.4C). We conclude that the severe weakening of the chemical synaptic connections produced by knocking down Hm-inx1 during electrical synaptogenesis was maintained long after embryogenesis was complete.

At the beginning of chemical synaptogenesis, P cells were electrically coupled to AP cells (Fig. 2.5A) through a connection with a coupling strength of about 1.5 - 2.5 mV/nA, which gradually waned over a day or two to a lower value (~0.5 mV/nA) that persisted into adulthood (Fig. 2.5A). During the same period, the number of cells that were dye-coupled to the P cell increased. To determine whether electrical synapses are formed after the effects of siRNA injection wear off, we injected siRNA into P cells at 48-50% ED and assayed for dye-coupling and coupling strength either at 70% ED, or at juvenile stages (21 to 35 days post injection). At all stages tested, the difference between the number of cells dye-coupled to inx1-siRNA-treated P cells was not significantly different from controls (Fig. 2.5B). The locations of leech neurons are sufficiently variable that we could not determine whether every dye-coupled cell was identical with those dye-coupled in control ganglia, but the patterns were at the least very similar. In addition, the electrical coupling strength between treated P cells and AP cells was not significantly different from control values both at 70% ED and in juveniles (Fig. 2.5C). Thus, by these two measures, electrical coupling returned as the effect of the siRNA waned, although chemical synaptic connections remained significantly diminished (Fig. 2.4).

Figure 2.4 Blocking electrical synapses perturbed chemical synaptogenesis

A. EPSPs in an AP cell in response to single spikes in a P cell from the same ganglion; the P cell spike occurred at the time marked by the grey bar on the AP recordings. AP cells were held hyperpolarized to silence their tonic firing. Data shown are from a single trial. Colors in the cartoon indicate how the P cell was treated. B. EPSP amplitudes recorded in AP cells in response to a P cell action potential. When a P cell was treated with siRNA prior to the onset of chemical synaptogenesis, it produced much smaller EPSPs in the AP cell, both one week after treatment (70% ED) and 3-5 weeks after treatment (juvenile stage) (ANOVA, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). C. AP cells responded to a spike in an untreated P cell by firing a time-locked action potential ~80% of the time. Treating the P cell with siRNA greatly reduced the incidence of time-locked action potentials in APs following anode-break spikes in the P cell, both at 70% ED and in the juvenile stage (ANOVA, ** $p < 0.01$, *** $p < 0.001$). Numbers within each bar in panels B and C indicate the number of preparations of each type.
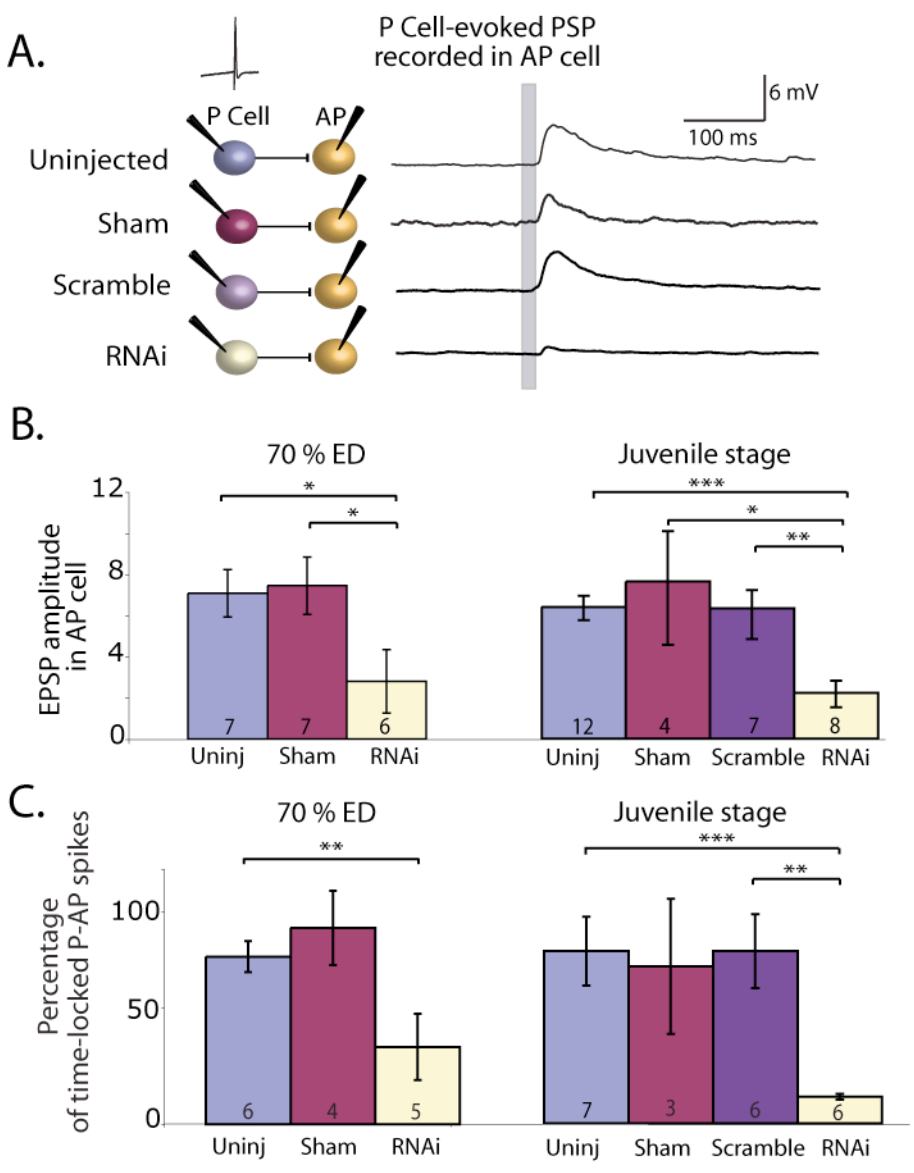
Figure 2.5 The electrical synapse between the P and AP returned to normal following RNAi

A. Measure of the strength of the electrical synapse and dye-coupling between the P cell and the AP cell. In black, the coupling strength between P and AP is strong early in development and wanes, but persists into adulthood. In grey, the number of neurons that are dye-coupled to a P cell increases to a steady state at the juvenile stage. B. The number of neurons dye-coupled to P cells injected with siRNA returned to normal by 70% ED, i.e., 1 week after treatment, and persisted in juveniles. Blue bars indicate the number of somata dye-coupled to uninjected P cells, red to sham injected P cells (injected with the vehicle Alexa 488), and pale yellow to P cells injected with active targeted siRNA. C. Electrical coupling strength between the P cell and the AP cell fell within the normal range 1 week after treatment and persisted into the juvenile stage. Blue bars indicate the coupling strength from an uninjected P cell to the AP cell, red sham injected P cells (injected with the vehicle Alexa 488), purple scrambled siRNA injected P cells, and pale yellow P cells injected with active targeted siRNA.

A.

B.   70% ED   Juvenile stage

C.   70% ED   Juvenile stage
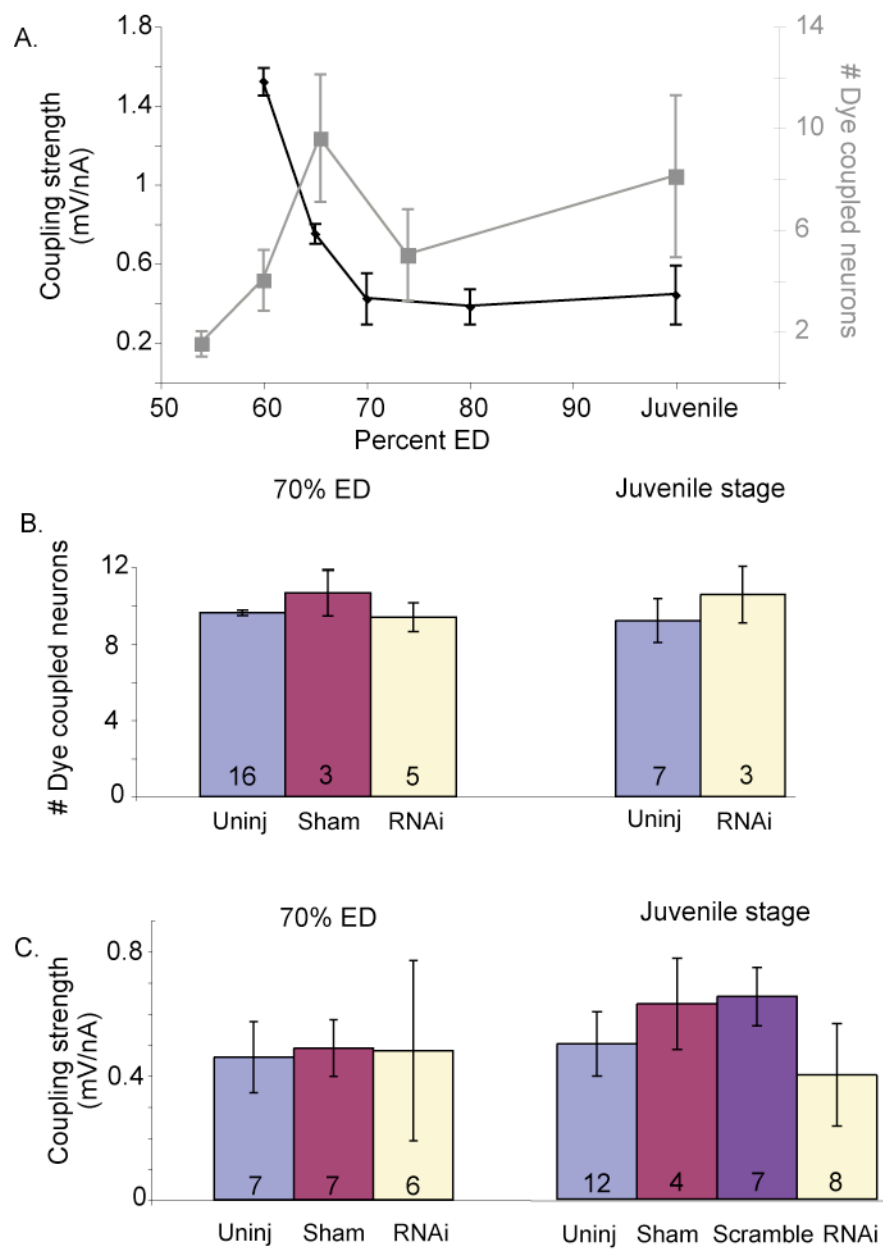
Effect of disrupted chemical synaptogenesis on normal behavior

To determine whether the modifications of chemical synapses formed by a treated P cell affected behavior, we used the local bending response. Local bending is a segmental behavior (i.e., each segmental ganglion contains the full complement of identified neurons required to produce local bending in the ganglion's home segment) that can be elicited by stimulating a single P cell (Lewis and Kristan, 1998). Applying moderate pressure to the body stimulates one or two of the four P cells in a ganglion (depending on the location of the touch), which activates a single functional layer of interneurons that, in turn, activate appropriate motor neurons. In local bending, longitudinal muscles around the site of the touch contract, bending the segment away from the touch. Local bending can be readily generated in a reduced (body-wall) preparation consisting of a single ganglion connected to a piece of body wall, which can be used both to stimulate the P cells and to record the contractions of the muscles (Baca, et al., 2005; Lockery and Kristan, 1991). In embryos at 50% ED, we injected inx1-siRNA into a P cell that innervates a dorsal quadrant of the body wall (Pd) and then allowed the embryos to develop into juveniles. We made a body-wall preparation from the segment containing the treated Pd cell and applied stimuli of different

intensities to the middle of its receptive field. As a control, we used an untreated P cell that innervated an adjacent, ventral quadrant (Pv) in the same segment (Fig. 2.6A). We made digital video recordings of the body wall movements made in response to the stimuli and used optic flow analysis to quantify longitudinal contractions (Baca, et al., 2005; Thomson and Kristan, 2006; Zoccolan and Torre, 2002) (Fig. 2.6B, C). In control animals, we observed that stimuli in the receptive fields of either Pd or Pv caused the expected longitudinal contractions, and as previously reported (Baca, et al., 2008; Baca, et al., 2005), stronger stimuli elicited stronger contractions (Fig. 2.6D). However, when we stimulated in the expected receptive field of the Pd cell that had been treated with inx1-dsRNA, we rarely observed local bending, and the contractions that did occur in this region were small and failed to increase with increasing stimulus intensities (Fig. 2.6D). In these animals, responses in the receptive field of the (untreated) Pv cell with a receptive field adjacent to the receptive field of the treated Pd cell elicited local bending that was indistinguishable from control animals, except at the highest force values, in which contractions were slightly attenuated (Fig. 2.6E).

There are two possible explanations for the lack of local bending response following siRNA treatment: chemical synaptic

transmission from the treated P cell onto local bend interneurons

remains greatly reduced at this advanced development stage, or the

sensory P cell is not effectively receiving and transducing stimulus

information from the periphery. To test whether RNAi treated P cells

were reacting normally to sensory input, we produced "hole-in-the-

wall" preparations (Baca, et al., 2008). Briefly, we produced the same

kind of body wall preparation shown in Fig. 2.6A, except that we

made the longitudinal incision along the dorsal midline, and made an

additional small incision along the ventral midline to expose the

segmental ganglion (Fig. 2.7A). This preparation leaves much of the

ventral skin and all of the dorsal skin functionally innervated. The

number and frequency of spikes elicited in a P cell by peripheral

stimulation depends on the force exerted, on the location of the

stimulus within the cells receptive field in the body wall and on the

stimulus duration (Lewis and Kristan, 1998). We recorded the number

of spikes generated by Pd cells in response to brief, 30 mN pokes

applied at the center of the cell's receptive field using a calibrated

tungsten wire (Fig. 2.7B). In addition to the uninjected and sham-

injected controls, this preparation provides an in-ganglion control: we

recorded the response of the untreated contralateral Pd cell to the

same stimulus applied in its own receptive field. The Pd cells that were

Figure 2.6 Stimulating siRNA-treated P cells failed to elicit a behavioral response

A. Body wall preparation used to test local-bend response. This is a 7-segment long piece of body wall in which only the segment containing the treated P cell remains innervated (the area between the dashed lines). To produce this preparation we made an incision along the lateral edge of the leech, opened the body wall and pinned it flat. This configuration allowed us to apply a tactile stimulus within the receptive field of the Pd (dorsal, yellow oval) or the Pv (ventral, white oval). B. Maximal observed longitudinal contraction following a stimulus in the receptive field of a Pd neuron. (Using an optic flow algorithm, the digital movie of the contraction was converted into a color-coded vector map). The point of the stimulus was located at the asterisk; red shades indicate motion downward toward the point of touch, and blue shades indicate motion upward toward the touch. In controls, longitudinal movement is centered on the asterisk indicating the stimulus is the focus of the body wall contraction. Colored rectangles below each image divide the body wall into quadrants, each of which is innervated by a single P cell: Pd indicates a dorsal quadrant; Pv indicates a ventral quadrant. These colors are also used in panel C. C. Amount of longitudinal motion in body wall preparations from three kinds of preparations: uninjected (blue line), one Pd injected with scrambled siRNA (purple line), and one Pd injected with active, siRNA (pale yellow line). To measure each response, we drew a rectangle two annuli wide around a region of interest (ROI) directly anterior to the stimulus. (Each leech segment consists of five rings visible in the skin, which are called annuli.) The amplitudes of the movements within the region, normalized to the anterior-posterior dimension of an annulus, were integrated to yield a local bend profile (Baca, et al., 2005). D. Variation in maximum displacement of the ROI (contraction strength) with increasing force. (ANOVA with Bonferonni post-hoc analysis, * $p < 0.05$, *** $p < 0.001$). E. Variation in ventral local bends in response to stimulation within the receptive field of a Pv. All colors are the same as in D; yellow bars show the results of <u>ventral</u> stimulation in a segment in which the <u>dorsal</u> P cell (Pd) was injected with siRNA.
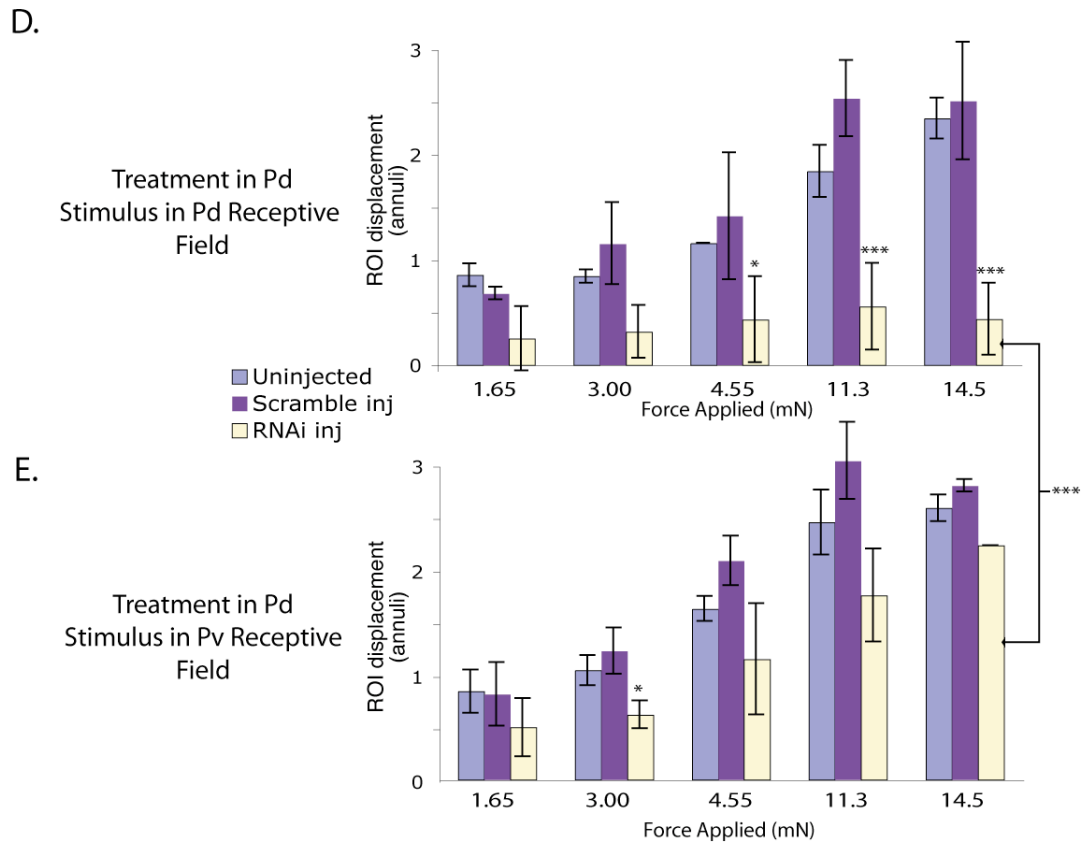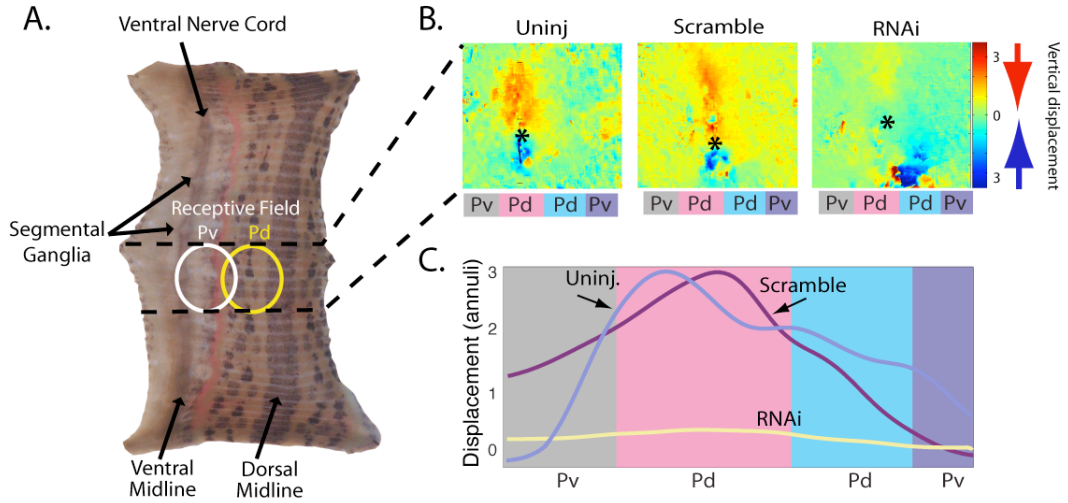
A. Ventral Nerve Cord, Segmental Ganglia, Receptive Field Pv Pd, Ventral Midline, Dorsal Midline

B. Uninj, Scramble, RNAi; Pv Pd Pd Pv; Vertical displacement

C. Uninj., Scramble, RNAi; Displacement (annuli); Pv Pd Pd Pv

D. Treatment in Pd Stimulus in Pd Receptive Field; ROI displacement (annuli); Force Applied (mN); Uninjected, Scramble inj, RNAi inj

E. Treatment in Pd Stimulus in Pv Receptive Field; ROI displacement (annuli); Force Applied (mN)
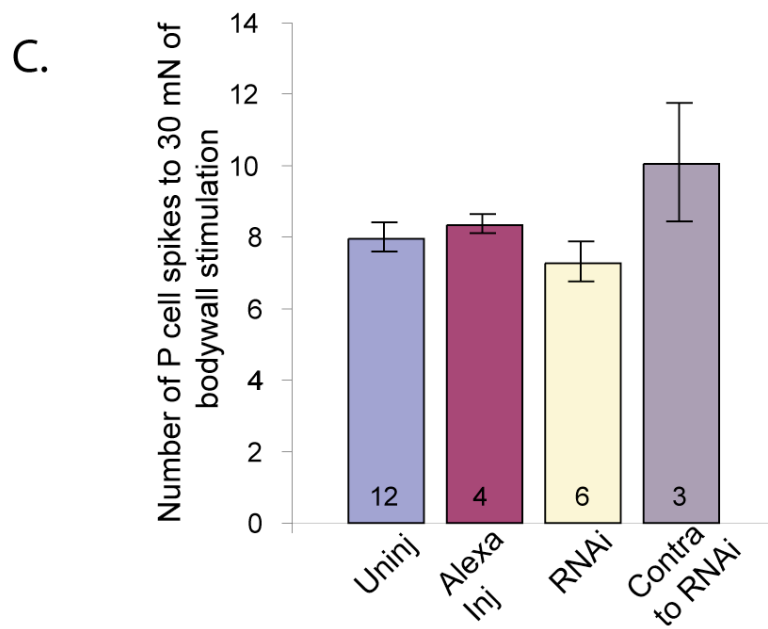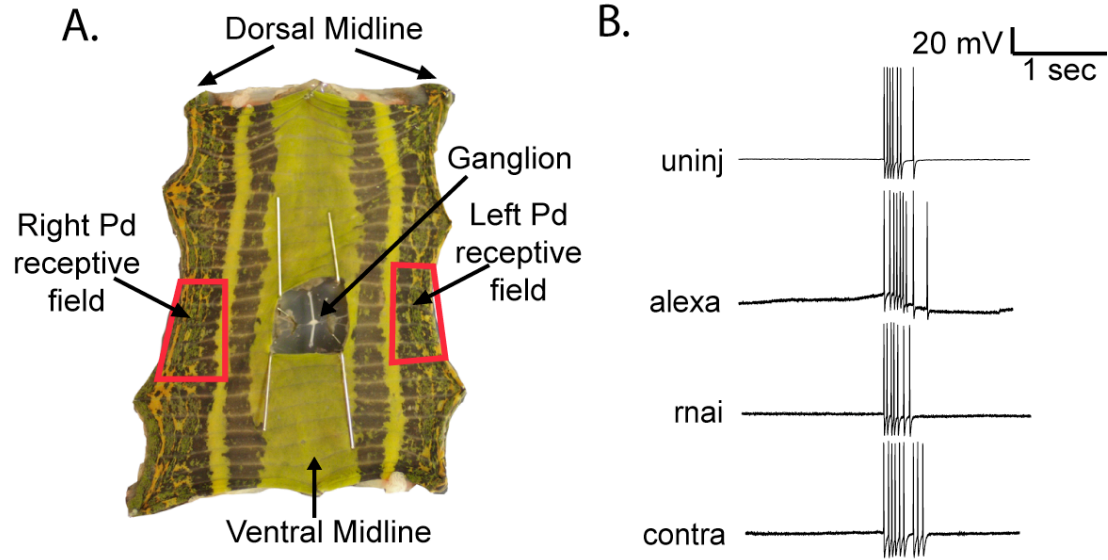
Figure 2.7 Treated P cells received normal sensory input

A. The "hole-in-the-wall" preparation showing the one functionally innervated segment in a 5-segment section of leech body wall. An incision was made along the dorsal midline, the piece of body wall was pinned flat, and a hole over the ganglion was held open by pins. The receptive fields of the two Pd cells are indicated (red boxes). B. Intracellular recordings from Pd cells in response to a brief (~ 500 msec) 30 mN stimulus in the cell's receptive fields. "Contralateral" refers to the in-ganglion control Pd cell located on the side of the ganglion opposite to the RNAi treated Pd cell.   C. Number of spikes in a 500 millisecond time-window following stimulation; the number of spikes elicited in a RNAi treated Pd cell was indistinguishable from controls. Color code as in Figures 4 and 5.

A. Dorsal Midline, Right Pd receptive field, Ganglion, Left Pd receptive field, Ventral Midline

B. 20 mV, 1 sec, uninj, alexa, rnai, contra

C. Number of P cell spikes to 30 mN of bodywall stimulation — Uninj (12), Alexa Inj (4), RNAi (6), Contra to RNAi (3)

treated with the siRNA at 50% ED produced responses indistinguishable from the responses seen in the uninjected, sham-injected, or contralateral P cell controls (Fig. 2.7C). We conclude that the P cell's entire chemical synaptic output, but not its sensory input, was perturbed as a result of transiently lacking electrical synapses during synaptogenesis.

Discussion

We used RNA interference, a technique invented in the 1990's for studying plants (Flavell, 1994) and fungi (Romano and Macino, 1992), and refined for use in animal models (Fire, et al., 1998). These early uses of RNA to block post-translational gene expression were revolutionary in furthering our understanding of RNA's regulatory role within cells. The early application of this technology, however, required transforming or applying the knockdown bluntly to the whole organism. The refinement of molecular genetic techniques in recent years, using temperature-dependent expression of the RNAi phenotype and promoter-driven expression of active RNAi components, has greatly improved the temporal and cellular specificity available to determine the necessity of particular gene

products (Brummelkamp, et al., 2002; Fortier and Belote, 2000; Napoli, et al., 2009) .

We have taken RNAi specificity a step further by directly injecting an RNAi- inducing agent into a single, identified neuron that we can follow through development into maturity. This method takes advantage of the known behavioral circuitry and development of individual leech neurons and combines it with the targeted gene knockdown of RNA interference to transiently decrease the function of electrical synapses. The combination provides unique control and allowed us to determine the behavioral relevance of a single molecule expressed in a single cell during a particular phase during the development of an organism.

Our results indicate that electrical synapses must be present in a leech mechanosensory neuron during the time of chemical synaptogenesis for it to make connections necessary to the production of local bending behavior. Even weeks after the effect of RNAi subsided and electrical coupling returned to normal, transmission through this behavioral circuit remained abnormal. Results such as these must lead to questions regarding the mechanism: what are electrical synapses doing that allow chemical synapses to form between appropriate neurons? Perhaps gap junctions play an

"active" role --e.g. transmission of some message or signal through the junction's pore--or maybe that they play a more passive role that does not depend on their ability to pass signals between cells. Electrical synapses are notable for their ability to pass electrical activity rapidly and with high fidelity and for their conductance to ions and small molecules. Coordinated electrical activity has long been correlated with the formation and refinement of chemical synapses (Cohen-Cory, 2002). Even if direct current passage from one neuron to another through electrical synapses is not important, electrical synapses have been shown to modulate neuronal excitability within entire circuits (Rela and Szczupak, 2005).

If electrical activity alone is not the synaptogenic factor, there are other candidates: ions such as calcium and calcium-releasing second messengers such as IP3 pass through gap junctions (Kumar and Gilula, 1996). These chemical species have been implicated as second messengers and synaptic transcriptional activators and could play a role in forming functional synapses.

In the second category are hypotheses based on the physical--rather than the functional--properties of electrical synapses. Recently it has been shown in mouse cortex that the adhesive quality of two hemichannels on apposing cells is required for neuronal migration

after neural differentiation (Elias, et al., 2007). This observation suggests that electrical synapses might facilitate chemical synaptogenesis simply by holding two neurons in close enough proximity to allow other synaptogenic processes to proceed.

Distinguishing among the possible mechanisms will require further experiments, but the ability to control expression of innexins one neuron at a time will permit further dissection of the relationship between gap junctions/electrical synapses and chemical synaptogenesis.

Chapter 3: Culturing leech embryos to study
synapses among motor neurons

Summary

To examine the details of synaptogenesis, we have studied the development of the neuronal circuit that produces local bending, a withdrawal response to moderate touch to the body wall. The local bending circuit has been characterized in adult leeches, and the component neurons are identifiable by half-way through embryogenesis. In intact embryos, the sensory neurons and many interneurons in this circuit can be readily manipulated because they can be directly exposed by making a small incision in the ventral body wall. However, most of the motor neurons in *Hirudo* are located on the side of the ganglion that faces the inside of the embryo, and previous attempts at culturing dissected embryos have remained healthy only for 2 - 3 days, thus making it impossible to manipulate them for long-term study *in vivo*. To address this problem we have developed a method of culturing dissected leech embryos that allows cultured embryos to develop stage-specific anatomic characters at the same rate as control siblings. Additionally, the features of

identified neurons, such as elaboration of central arbors, development of dye-coupling among neurons, and general ganglion morphology were very similar in cultured embryos and in their intact siblings. This method allowed us to study the development of synaptic connections among motor neurons using the methods that we previously used to study synaptogenesis of sensory neurons. Using the technique, we determined that the single-cell RNAi technique described in Chapter 2 effectively blocked electrical synapses between motor neurons of cultured embryos.

Introduction

The development of motor neuron circuitry has for over a century been studied in a variety of vertebrate and invertebrate species (Fredette and Landmesser, 1991; Harrison, 1988; Landmesser, 2001). Exploration of synaptogenesis by developing motor neurons has focused both on the peripheral neuromuscular junction, in which a motor neuron is presynaptic (Fox, et al., 2007; Loeb, et al., 2002; Nose, et al., 1992; Shen and Bargmann, 2003), and within the CNS, where motor neurons are largely post-synaptic (Frank and Westerfield, 1983). Motor neuron axons both extend and form synapses concurrently (Javaherian and Cline, 2005), which raises the question

whether these two developmental processes might interact, with each shaping the outcome of the other. In leech development, motor neurons extend their axons out of their ganglion later than mechanosensory neurons (Jellies and Johansen, 1995; Kuwada and Kramer, 1983), but within a ganglion electrical connections form among the motor neurons at least a day before mechanosensory neurons and interneurons make their first electrical connections (Jellies and Johansen, 1995; Kuwada and Kramer, 1983; Marin-Burgin, et al., 2006). Despite the general experimental tractability of leech embryos, it has proven difficult to perform a longitudinal study of synapse formation and refinement among motor neurons in leech embryos because the somata of most leech motor neurons lie on the dorsal surface of the ganglion, which faces the yolk inside each embryo. To access these cells for study, the yolk sac must be dissected open, pinned out, and have its yolk removed. Because the yolk sac membrane undergoes myogenic contraction for much of development, a non-anesthetized animal will often tear itself apart after only a few hours in the dish (Fig. 3.1).

Leech embryos are not the only animals that make very inconvenient movements: movements of embryonic flies, tadpoles,

Figure 3.1 Myogenic contraction of the yolk membrane destroys the embryo structure

This embryo was dissected at about 40-45% ED and pinned in a dish of normal leech saline. After dissection and pinning (T=0) the yolk membrane was pulled taught with the embryo clearly visible in the middle. After 1 hour, the edges of the yolk membrane had begun to contract, and some ripping was evident. Eight hours later, the membrane was more contracted and was ripped at all the points of pinning. In addition, the embryo had come free from 2 of the pins and was beginning to curl in on itself. Unpinned embryos roll up tightly and form an impenetrable cylinder.

T = 8 Hours

T = 1 Hour

T = 0 (at dissection)

yolk sac

germinal plate

pin
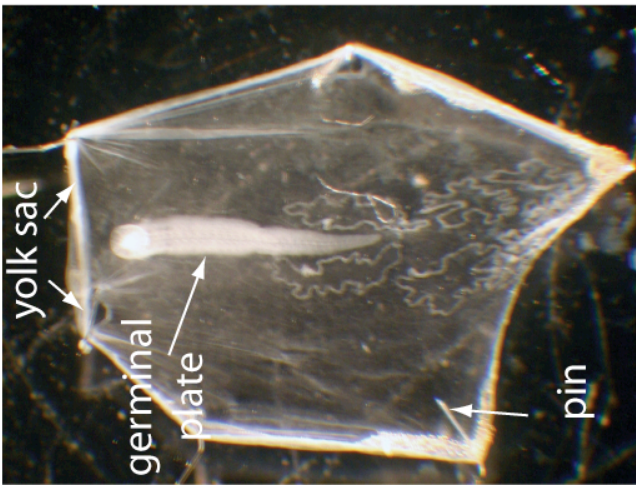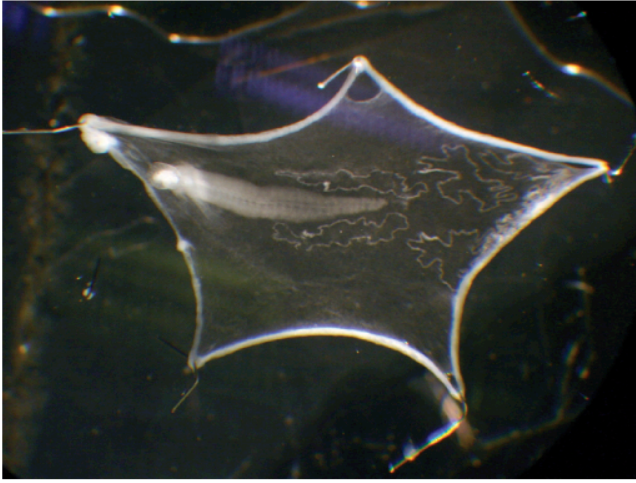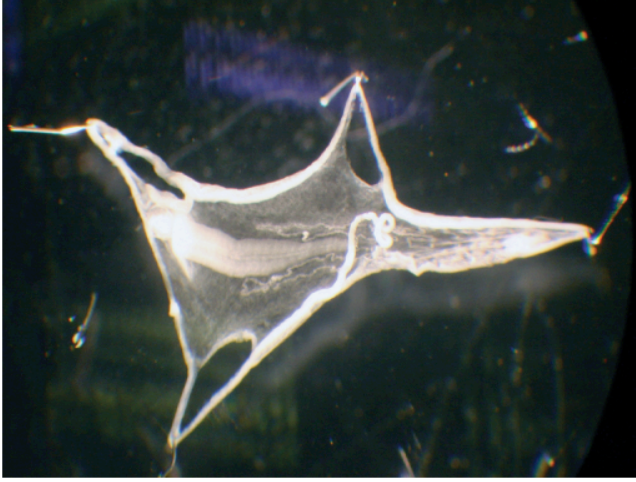
and fish produce recording artifacts (Hewapathirane, et al., 2008; Jasanoff and Sun, 2002; Langenberg, et al., 2003; Sun, et al., 2003).

To date, the longest reported time for maintaining leech embryos in organotypic culture has been 2 days (Song and Zipser, 1995). We have used ideas from other systems and developed an agarose embedding technique that allows us to perform experiments on individual identified motor neurons in the leech embryo and to assay their development as many as 4 days later.

Methods

Animals

Leech embryos were produced in our breeding colony of *Hirudo verbana*. They were released from their cocoons and were then held at 20–24°C in "embryo water," sterile-filtered Arrowhead spring water (Arrowhead Water, Brea, CA) with 32 micromol of $MgCl_2$ and 40 micro mol of $CaCl_2$ added per liter.

Staging

We determined the embryonic stage of each embryo using external morphological features that run from 0% (egg deposition) to 100% of embryonic development (%ED) (Reynolds, et al., 1998).

Development proceeds at about 3% ED per day at 20°C. All experiments were performed in ganglia from the mid-body of the animal (segments 7–15) to minimize the effects of the anterior–posterior gradient of development in the leech; ganglia in this region differ by no more than 1% ED (Reynolds, et al., 1998).

Tissue Cultured Embryos

Embryos at 50% ED (for morphology and development) or 60% ED (for siRNA injections) were selected from our breeding colony. A solution of 2.5% agarose in L-15 culture medium (Invitrogen) was heated until the agarose dissolved, and UV sterilized 60mm culture dishes were half-filled with the agarose solution, which was allowed to solidify at room temperature in the UV hood. The flask holding leftover agarose mixture was held at 70°C to keep the solution liquid. Dissection tools and pins were sterilized for 15 minutes in 70% ethanol. Leech saline containing 8% ethanol was added to the half-filled dishes and embryos were transferred into the saline/ethanol solution. The embryos were pinned anterior and posterior with the dorsal side facing up, a longitudinal incision was made into the yolk sac, and all yolk was removed. The embryos were then pinned with the inside of the germinal plate facing up, providing access to the neurons on the

dorsal side of the ganglia. The embryos were then embedded by

slowly dripping the leftover agarose solution until they were covered.

The dish was rinsed once more with 8% ethanol in saline. which was

replaced with embryo medium (in 1 liter of L-15 medium add 1%

penicillin-streptomycin (Gibco), 1% L-glutamine (Gibco), 100 uM

vitamin C (Sigma), 0.15% glucose (Gibco), and 1% ITS+ (Gibco), and

dilute with 50% embryo water and add 2% heat-inactivated FBS

(Gibco)); the medium was changed daily. The agarose covering the

embryo (the "agarose cap") is readily removed to allow access to the

embryo.

**Morphology of ganglia and neurons**

The developmental progress of ganglia in cultured embryos

was compared to those in intact embryos. To determine the area of

each ganglion, we captured images using an AxioCam Hrc (Zeiss)

and AxioVision software (Zeiss), defined region of interest as the

perimeter of the ganglion, and we calculated the area of the region

using ImageJ software (http://rsbweb.nih.gov/ij/).

Developmental progress of individual identified neurons was

evaluated by observing the overall shape of a neuron filled with

fluorescent dye, determining the extent and number of neurites, and

**Figure 3.2 Cartoon schematic of embedding method**

A. A leech embryo at ~47.5% ED. This embryo was injected with a contrast-enhancing dye under the germinal plate. The yolk (yellow-brown) is clearly visible behind the dye. B. An incision was made along the dorsal midline of the embryo, the yolk was removed, and the embryo was pinned out onto a plate of 2.5% agarose made with L-15 medium. C. After experimental manipulation of the dorsal neurons, the remaining warm L-15 agarose was dripped on top of the pinned out embryo, forming a cap that held the embryo relatively immobile and that could be removed later.
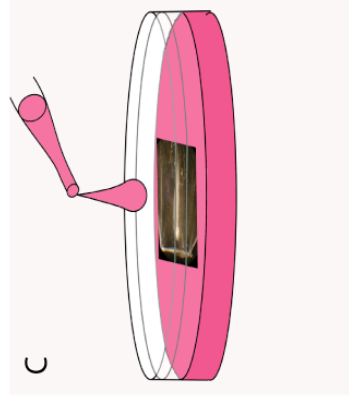
Embryonic Culture

A
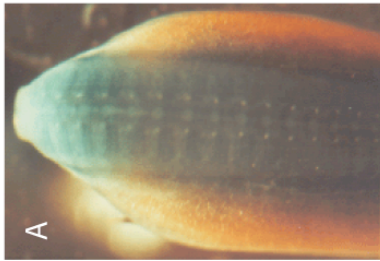
Whole Embryo
~47.5 % ED
(photo courtesy K. French)

Dissect, remove
yolk and pin

B
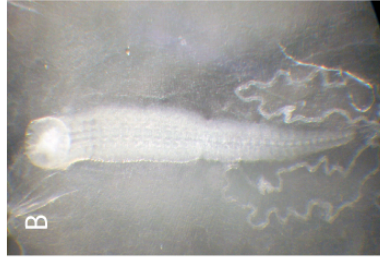
Perform manipulation
and drip agarose over
embryo

C

Pinned Embryo
Embedded in 2.5% Agarose

counting the branch points. To fill neurons in cultured embryos with a dye, we removed the agarose cap from the culture dish, added saline/8% ethanol, and impaled individual identified neurons on the dorsal surface of the ganglion with a microelectrode containing 2.5% AlexaFluor 488 dextran (10,000 mw, Invitrogen) (electrodes backfilled with 3M Potassium acetate). The dye was passed into the neurons with 500 millisecond, depolarizing, 1nA current pulses (50% duty cycle) using an Axoclamp 2A amplifier and pClamp software (Molecular Devices). The injection was monitored using fluorescence images obtained throughout the filling process using an AxioCam Hrc (Zeiss) and AxioVision software (Zeiss) set at standardized acquisition settings. Once the average somatic pixel intensity was greater than 80% maximum value, the injection was stopped. The dye was allowed to diffuse for 45 minutes, and the tissue was then fixed using 4% paraformaldehyde in 0.1 M phosphate buffer for 2 hours at room temperature. The fixed embryos were rinsed 6 x 10 minutes with PBS, and dehydrated in an ethanol series. The tissue was cleared using methyl salicylate and mounted in DePeX mounting medium (Electron Microscopy Sciences, Hatfield, PA, USA). We used a Bio-Rad confocal scanning microscope to produce images of the injected neurons observed the morphology of projections and branching.

Dye coupling

Injections were made as above, but the electrodes were filled with 2.5% Alexa Dextran 488 and 2.5% Neurobiotin (Vector Labs). The mixture was allowed to diffuse for 45 minutes and then the tissue was fixed overnight in 4% paraformaldehyde at 4° C. The next day we rinsed them 6 x 10 minutes in PBS, and the tissue was incubated overnight at 4° C in Streptavidin conjugated to Cy3 (Jackson ImmunoResearch) at 2μg/mL in PBX (0.3% Triton X-100 in PBS). The embryos were again washed 6 x 10 minutes in PBS, dehydrated in an ethanol series, and mounted Gurr DePeX mounting medium (Electron Microscopy Sciences, Hatfield, PA, USA).

Standardized acquisition settings were used to obtain confocal Z-stack images of the preparations. The stacks were then processed using ImageJ to determine the ganglion's average pixel intensity and the standard deviation. A Look-Up-Table LUT was created that displayed pixels with intensities over 1 standard deviation (SD) above the mean. An intensity greater than or equal to the mean + 1SD was set as the criterion for containing Neurobiotin and, therefore, being dye-coupled to the injected neuron. We then examined the z-stack frame by frame and used this criterion to count the coupled neurons.

siRNA injections

siRNA fragments targeting innexin-1 mRNA (discussed in the previous chapter) were diluted to 10 – 15 µM in 1.5% AlexaFluor 488 Dextran and then wicked into sharp microelectrodes that had resistances of 60 – 80 MΩ if filled with 3M KAc. In embryos at 60% ED we impaled identified motor neurons DI-1, DE-3, or VE-4 using transmitted light. The siRNA / AlexaFluor mixture was pressure injected (100 psi) at ~ 1 Hz using a Picospritzer II (General Valve Corporation); progress of the injection was monitored using fluorescence optics. Fluorescence images were obtained through the filling process using an AxioCam Hrc (Zeiss) and AxioVision software (Zeiss) set at standardized acquisition settings. Once the average somatic pixel intensity was greater than 80% maximum value, the injection was stopped.

Determination of electrical coupling strength

We removed the agarose caps covering the embryos and rinsed the embryos with cold, normal leech saline (Muller, et al., 1981). We identified the previously injected cells based on the AlexaFluor 488 that remained within their somata.

We recorded from neuronal somata of both of the paired motor neurons DE-3 using sharp microelectrodes that had resistances of 50–80 M when filled with 3 M potassium acetate. Because embryonic neurons are extremely sensitive to osmotic changes, the electrodes were filled with 120mM potassium gluconate, which is nearly iso-osmotic with the external medium, producing a resistance of 200–600 megOhm. To prevent photo-toxic damage, cells were illuminated very briefly (1 s) during recordings. We measured the strength of an electrical synapse by passing multiple current steps into one cell and recording the voltage responses in the coupled cell. For the non-rectifying electrical synapses between the two cells DE-3, these steps were passed alternately into both cells of the pair. To compute the coupling strength, we divided the voltage response in the coupled cell by the stimulus current using a custom program written in MATLAB (The Mathworks, Natick MA); the slope of this line is the coupling strength. This quantity is also called the "transfer" or "mutual resistance" (Bennett, 1966), but we prefer to use the less arcane term "coupling strength;" we report our results in units of millivolt postsynaptic per nanoampere presynaptic, rather than in MΩ.

Statistics

t tests were used to evaluate statistical significance. All error bars are standard errors

Results

Ganglia and neuronal morphology developed normally in culture

Although the overall length and width of cultured germinal plates remained constant during their time embedded in agarose, they did acquired many of the same stage-specific morphological characteristics (e.g. slight dorsal fusion at the anterior end, loosening of the larval nephridia (Reynolds, et al., 1998) as their uncultured siblings that increased in both length and width. Similarly, although the ventral nerve cord failed to elongate normally during 4 days in culture, the ganglia of cultured embryos continued to develop at the same rate as ganglia in their uncultured siblings (Figure 3.3). The condition of cultured embryos deteriorated after 5 days. In embryos that were dissected at 50% ED, this time point corresponds to a stage of 65% ED in the uncultured siblings--a time when the right and left sides of normal embryos have almost completely fused.

We wanted to know whether the motor neurons on the dorsal surface of the ganglion developed normally in a cultured embryo. Morphometric parameters such as soma size (Tavazoie, et al., 2005;

Zaidel, 1999), dendritic arborization (Ristanovic, et al., 2006; Sholl, 1953; Wong, et al., 2000), and primary axon length (Gabel, et al., 2008) have been used to characterize neuronal development. We chose to examine the developing morphology of the motor neuron cell DE-3, which excites longitudinal muscles in the dorsal region of its home segment (Muller et al., 1981). After culturing embryos for 2, 3, or 4 days, we filled cells DE-3 with AlexaFluor 488 and analyzed them as described in Methods. Based on their general appearance and complexity, filled cells in cultured embryos were indistinguishable from the cells DE-3 in non-cultured siblings (Figure 3.4). To avoid using measures that were affected by vagaries of stretch imposed during pinning, we measured the area of the soma and the number of neurites to compare cells in cultured and non-cultured embryos.

Electrical synapses developed normally in culture

Previous work revealed that in the period during which we cultured leech embryos (from 50% ED to 62%ED),cells DE-3 become connected via electrical synapses (Marin-Burgin, et al., 2005). To determine if culturing the embryos modified the process of electrical synapse formation, we used two assays: dye-coupling and electrophysiological determination of coupling strength.

Figure 3.3 Ganglion area was similar in cultured and uncultured embryos

The size of the ganglia in cultured embryos increased at the same rate as ganglia in uncultured siblings. Embryos were cultured at about 50% ED, and removed from culture after 2, 3, or 4 days. The ganglionic area was measured as described in Methods.
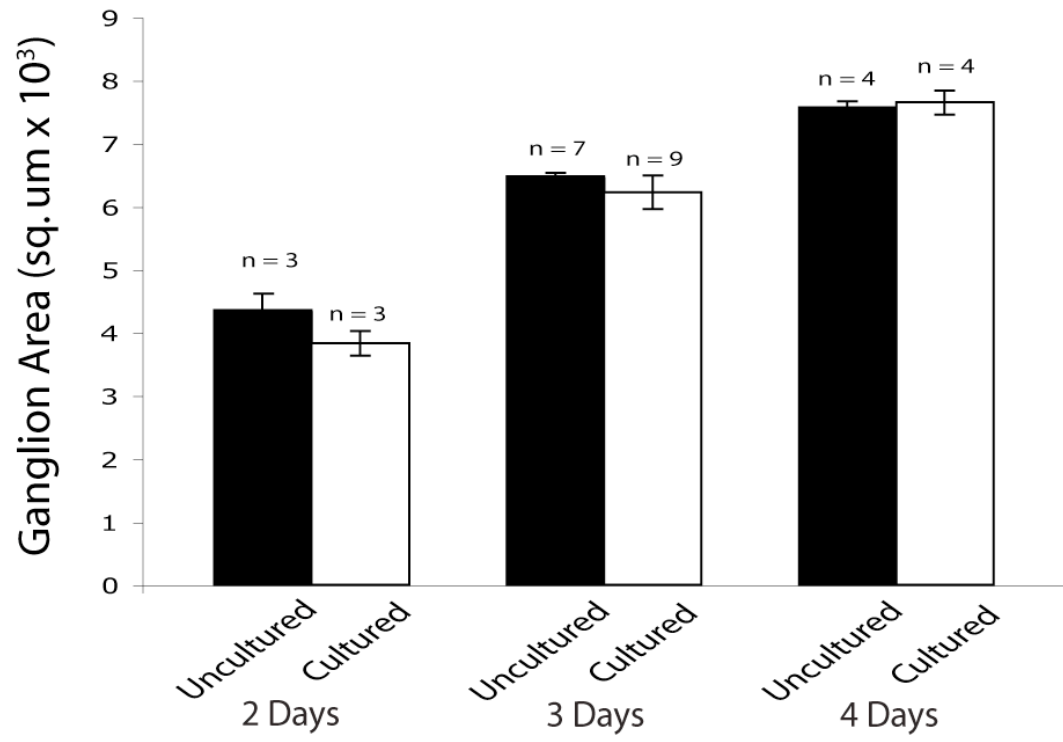
Figure 3.4 Motor neuron cell DE-3 morphology was unaffected by culturing

A. Collapsed confocal z-stack images of motor neuron cell DE-3 in sibling embryos. After two days, the cultured embryo's cell DE-3 extended a process across the ganglion midline, elaborated branches extensively on the side contralateral to the soma, and projected out to the body wall. The uncultured sibling looked similar. On the third day, both the cultured and uncultured embryonic cells DE-3 had begun to make ipsilateral neurite elaborations. The fourth day showed extensive ipsi- and contra- lateral elaborations in both the cultured and uncultured cells 3. (Notice that the cells shown at different time points are from different embryos.) B. Measurements of soma area were taken of three different sets of embryonic cells DE-3 at 2, 3, and 4 days into the experiment (n = 4 in all cases). There was no difference between the cultured and non-cultured embryos in soma size or rate of somatic growth as the embryos progressed from 50% ED to 62% ED. Similarly, the number of neurite processes on the side of the ganglion ipsi-lateral to the soma and extending anteriorly from the main neurite increased equally in cultured and non-cultured embryos as development progressed.
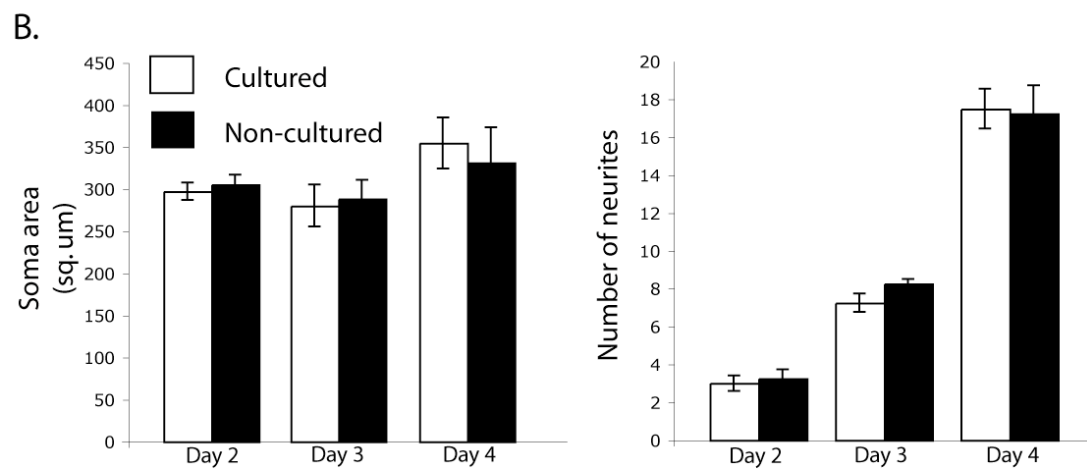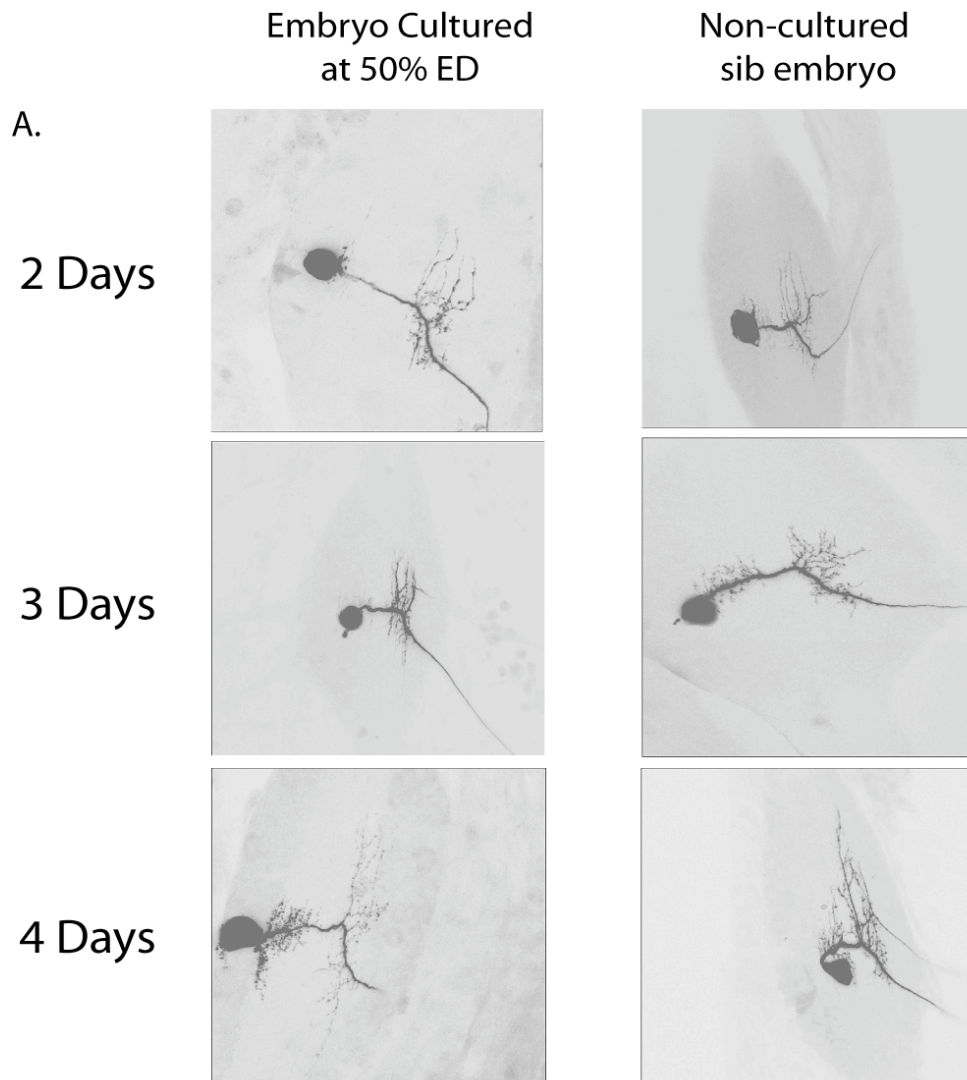
Embryo Cultured
at 50% ED

Non-cultured
sib embryo

A.

2 Days

3 Days

4 Days

B.

electrical synapse formation we used two assays: dye-coupling, and electrophysiological determination of coupling strength. Neurobiotin is a small molecule that passes through electrical synapses; it can be injected into one neuron and processed using an avidin-conjugated fluorophore to reveal cells coupled to it by gap junctions (Fan, et al., 2005; Peinado, et al., 1993). We injected Neurobiotin into cells DE-3 in embryos that had been held in culture for 2, 3, or 4 days and compared the number of dye-coupled cells in cultured embryos with the number in non-cultured siblings. We observed that in both groups, the number of dye-coupled neurons increased during the experimental period, but there was no difference between the cultured and non-cultured groups (Figure 3.5A, B).

Previous work determined that cell DE- 3 is electrically coupled to its contralateral homologue (Ort, et al., 1974). To test whether the DE-3 cells remained electrically coupled in cultured embryos, we removed the agarose caps from embryos that had been in culture for 4 days, simultaneously impaled both of the cells DE-3, and passed a series of hyperpolarizing current steps into each of the neurons three times while recording from the other. We confirmed that this is a non-rectifying electrical synapse, because the coupling in one direction was not different from the coupling in the other direction. We
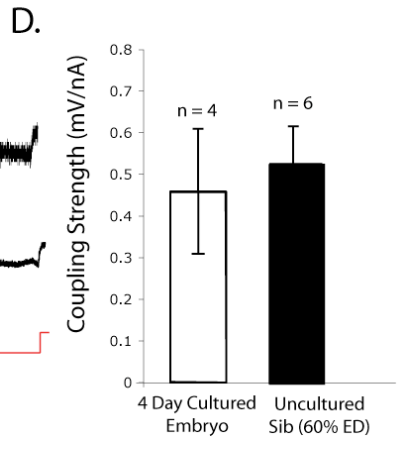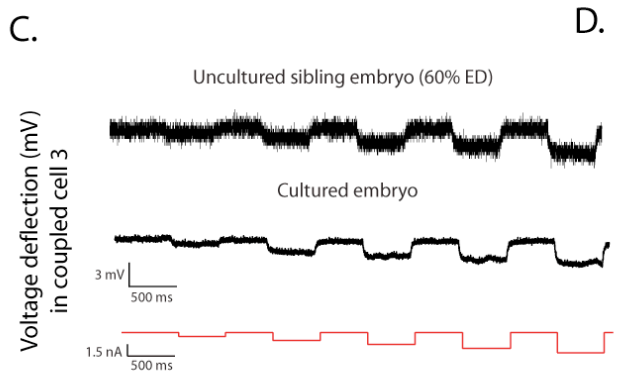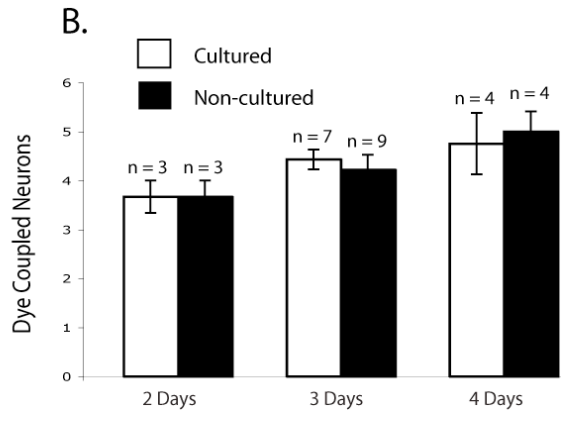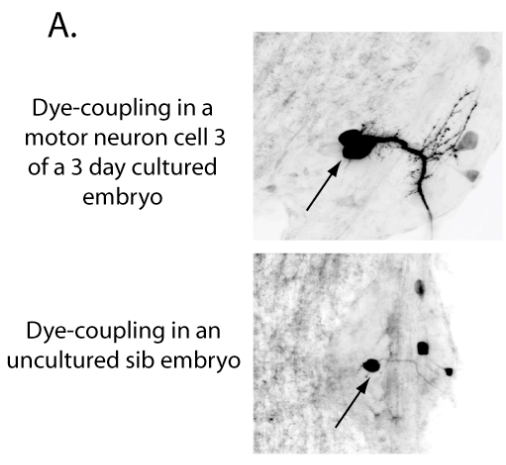
averaged the coupling strength values for each animal and compared them to values obtained from non-cultured siblings. We found that the electrical coupling strength between cells DE-3 was unaffected by culturing (Figure 3.5 C and D). Thus, morphologically and physiologically, the nervous system of a cultured embryo was indistinguishable from a non-cultured sibling. From these experiments, we conclude that leech motor neurons develop normally in culture for at least 5 days.

Using siRNA to block electrical synapses in motor neurons

We have successfully used RNA interference to block the function of electrical synapses in leech embryos (Chapter 2), but initially we studied only cells with somata on the ventral face of the ganglia, which is accessible through the embryonic body wall. We next wanted to determine if this tool could be used to study the development of embryonic motor neurons in cultured germinal plates. All motor neurons in *Hirudo* embryos contain high levels of innexin-1 mRNA (Dykes and Macagno, 2006), and we have generated small-interfering RNA (siRNA) that targets this sequence. We cultured *Hirudo* embryos at 50% ED. Before the final agarose cap was placed over the embryos, the siRNA in AlexaFluor 488 was injected into one

Figure 3.5 Electrical synapse physiology is unaltered by embryo culture

A. Representative collapsed confocal stacks of cell DE-3 after 3 days in culture and in an age-matched sibling. Cell DE-3 (black arrows) was injected with Neurobiotin and processed with streptavidin-Cy3 to reveal the dye-coupled neurons (dark somata). B. The number of cells dye-coupled to cells DE-3 in cultured embryos was not significantly different from non-cultured controls. C. Raw data of an electrophysiological determination of coupling strength between cell DE-3 and its homologue. The top trace shows the voltage deflection in the "post-synaptic" cell DE-3 in an intact embryo when current steps of different amplitude were injected into the "pre-synaptic" cell DE-3. The middle trace shows the voltage deflection in cell DE-3 of a cultured embryo (4 days) under the same conditions, and the bottom trace shows the current protocol delivered to the "pre-synaptic" cell DE-3. D. There was no difference in the coupling strength between cells DE-3 when cultured and intact embryos were compared.

A. Dye-coupling in a motor neuron cell 3 of a 3 day cultured embryo

Dye-coupling in an uncultured sib embryo

B.

C. Voltage deflection (mV) in coupled cell 3

Uncultured sibling embryo (60% ED)
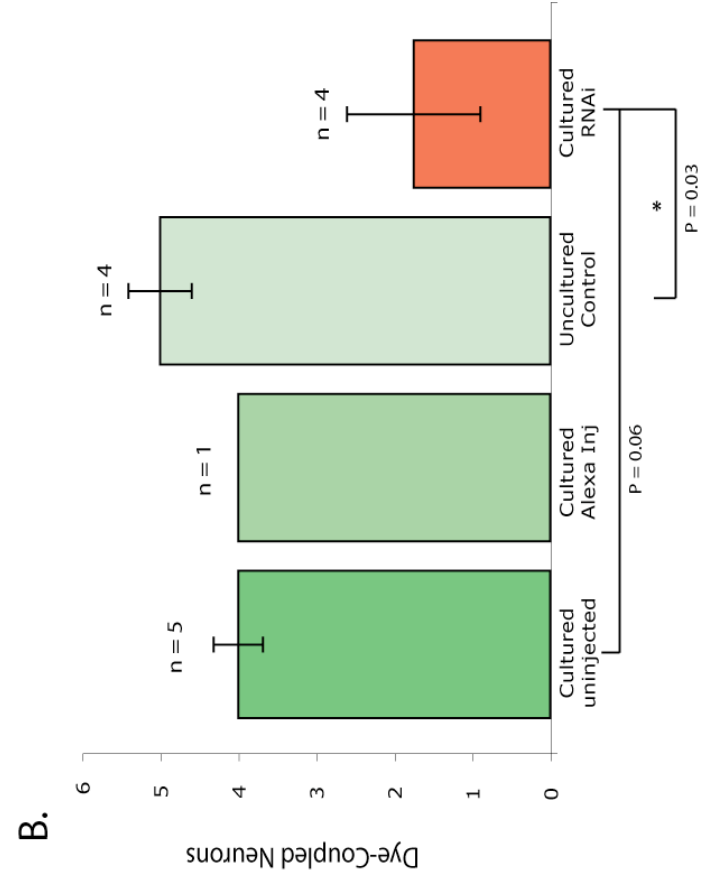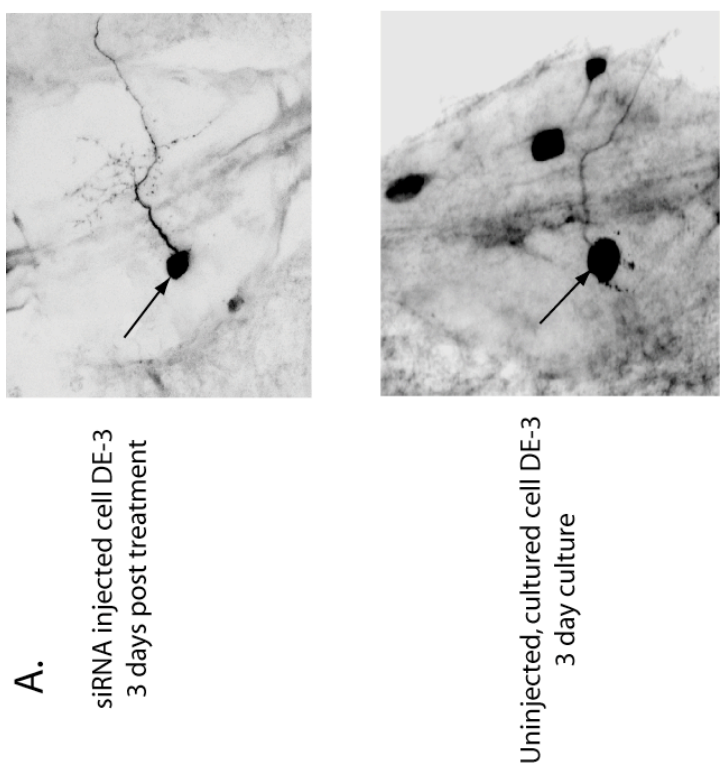
Cultured embryo

D.

cell DE-3 in each embryo. We then covered the embryos with agarose and maintained them as before. After 3 days in culture we uncovered the embryos and injected the same cell DE-3 (which we recognized because it still contained AlexaFluor 488) with Neurobiotin to reveal electrical coupling (see Methods). We found that the number of dye-coupled cells was noticeably smaller following RNAi treatment (Figure 3.6 A).

Discussion

Until recently, manipulating the development of leech motor neurons and then observing the results for more than a day or two has been impossible because the somata of most motor neurons are located deep in the germinal plate. We have successfully developed a culture technique that allows neurons to develop normally for as long as 4 days after the embryo is dissected, which is long enough to follow the events of synaptogenesis among neurons in the ventral nerve cord. Embedding an embryo in agarose and maintaining it in culture medium solves the problems associated with the myogenic contraction of the yolk sac while supporting normal motor neuron development in unanesthetized animals. Morphometric characters

Figure 3.6 siRNA against innexin-1 blocked electrical synaptic formation

A. siRNA to block the formation and function of electrical synapses in embryos at 50% ED. The top image shows Neurobiotin labeling after 3 days in culture in an embryo in which one cell DE-3 was treated with siRNA. The lower image shows cells dye-coupled to a cell DE-3 in a cultured sibling embryo that was cultured for 3 days, but not treated with siRNA. The dye-coupling pattern in this cultured was typical of cell DE-3 in intact embryos at this stage. Arrows indicate cells DE-3. B. The number of dye-coupled neurons was significantly lower in the siRNA treated group than in untreated controls (Students *t* Test).

A.

siRNA injected cell DE-3
3 days post treatment

Uninjected, cultured cell DE-3
3 day culture

B.

Dye-Coupled Neurons

n = 5   Cultured uninjected

n = 1   Cultured Alexa Inj

P = 0.06

n = 4   Uncultured Control

*

P = 0.03

n = 4   Cultured RNAi

indicated that ganglia in cultured embryos grow normally, and

neurons developed their processes at the appropriate times.

Functionally, motor neurons formed electrical synapses at the normal

time and with an appropriate number of partners.   These advances

allowed us to determine the effect of a useful molecular tool, RNA

interference, on motor neurons and revealed that injecting siRNA

targeted to innexin-1 (the protein that forms gap junctions in most

leech neurons) into motor neurons blocked the formation of electrical

synapses among these neurons, as it does when this siRNA is injected

into pressure-sensitive P cells (see Chapter 2). This finding will enable

many possible directions of study. For example, it will permit a

comparison of rectifying and non-rectifying electrical synapses.

Among the motor neurons on the dorsal face of the ganglion, it is

known that there is an electrical synapse connecting cell DI-1 (an

inhibitory motor neuron that innervates dorsal longitudinal muscle in

the body wall) and cell VE-4 (an excitatory motor neuron that

innervates ventral longitudinal muscle) (Kristan, et al., 2000).

At this junction, depolarizing current passes from cell VE-4 to cell DI-1,

but not from cell DI-1 to VE-4; hyperpolarizing current passes from cell

DI-1 to cell VE-4, but not the other way. Rectifying electrical synapses

have been discovered in a large number of species and locations,

and the mechanism that allows a rectifying electrical synapse to preferentially pass current in one direction, but not the other has yet to be determined. One hypothesis with some experimental support is that the gap junction hemichannels in opposing cell membranes are made up of differing subunits. Since we know that innexin-1 is present in all neurons, our culture technique, in conjunction with RNA interference to block innexin-1 based electrical synapses, will provide an excellent set of tools to directly test this hypothesis.

Chapter 4: Peripheral cues and neuronal identity

Summary

We are interested in how embryonic neurons acquire their final identity, including the synapses and morphology that are appropriate to each type of neuron and that permit an organism to interact with its environment and behave effectively. Many types of factors have been shown to play an important role in shaping the nervous systems of both vertebrates and invertebrates, including extracellular signals that a neuron encounters while growing (Bentley and Caudy, 1983; Oakley, et al., 1997; Sperry, 1963; Wenner and Frank, 1995; Zlatic, et al., 2009). During embryonic development, axons that extend into peripheral tissue may encounter many different cues that affect their final phenotype.

We are using embryonic leeches to explore the role played by peripheral tissue in determining the synaptic connectivity and morphology of neurons that send axons out of the ganglia. Because the ventral nerve cord is located along the ventral midline and leeches develop essentially as a 2-dimensional germinal plate, as the

axons of motor neurons extend out of a ganglion, they first encounter ventral tissue, then dorsal tissue. To determine whether these axons respond to signals in the body wall as they extend from the ganglion, we have transplanted dorsal tissue into ventral territory, forcing ventral-innervating neurons to encounter an inappropriate tissue.

We found that when tissue transplants were performed at 48% ED, the tissue retained its original fate and did not regulate to match its new environment. We saw that neuronal axons grew into the transplanted tissue and formed functional synapses. These developing motor neurons formed electrical synapses with other neurons, but the number of coupled neurons was variable, with no consistent pattern. These studies are as yet inconclusive, but they provide the groundwork for future studies of motor neuron development.

Introduction

During the development of the nervous system a growing neuron integrates a number of factors--such as electrical activity (Akaaboune, et al., 1999; Borodinsky, et al., 2004), the neuron's temporal "birthday" (Angevine and Sidman, 1961), and a variety of peripheral cues (Oakley, et al., 1997; Smith and Frank, 1987; Wenner

and Frank, 1995)--that influence the final identity of the neuron. The

role of peripheral cues has been studied in multiple species: *Xenopus*

(Hunt and Jacobson, 1972; Smith and Frank, 1987) chick (Summerbell

and Stirling, 1981), mice (Vrieseling and Arber, 2006) and the medicinal

leech, *Hirudo* sp. (Baptista, et al., 1990; Johnson, et al., 2000).

Leeches are annelid worms and therefore have a segmented

body. The leech central nervous system consists of a head brain, a tail

brain, and a ventral nerve cord made up of 21 ganglia that are

connected along the cord by connective nerves. Each ganglion

contains approximately 400 neurons (Macagno, 1980), which are

nearly the same in each of the 21 ganglia. In general, a ganglion

receives much of its sensory information from the body segment in

which it is located, and the motor neurons in the ganglion control

muscles exclusively in the body wall of that segment. Thus, ganglia

exert a great deal of local control, although information travels

through the nerve cord along the connective nerves as well. Each

identified motor neuron innervates muscle fibers in a specific portion of

the body wall. Two of the ganglia are noticeably different from all of

the others, and these two (midbody ganglia 5 and 6) are located in

the segments that include most of the reproductive organs. Leeches

are simultaneous hermaphrodites, and in *Hirudo* the fifth midbody

segment contains the male reproductive ducts (the testes are distributed along several segments of the posterior body) while the sixth midbody segment contains the female reproductive system. In leech embryos, disrupting the connection between the periphery and neurons in the fifth and sixth midbody ganglia affects neuronal fate in those ganglia. When the nerve roots of either of these two ganglia are severed at about 50 - 60 % ED, disrupting the connection between the ganglion and the embryonic sex organs, a population of small neurons unique to these two ganglia (the peripherally-induced central, or PIC, neurons) fail to form (Baptista, et al., 1990).

Although the function of the PIC neurons is unknown, another class of leech neurons, the Retzius cells, also responds to peripheral influences. There are two Retzius cells in each ganglion, and the serotonin they release affects the behavioral activity of the leech (Willard, 1981) and the contraction of muscles in the body wall (Mason and Kristan, 1982). Retzius cells have 2 phenotypes. In most segments, they branch in the muscles of the body wall and just beneath the dermis (Lent and Frazer, 1977). In the reproductive segments (that is, midbody segments 5 and 6), the Retzius cells arborize densely in the walls of the reproductive ducts and organs (Jellies, et al., 1987), rather than in the body wall, and they display

unique central anatomy and physiological properties as well. When the embryonic reproductive ducts are ablated about half-way through embryogenesis, the Retzius neurons no longer develop their unusual properties; instead they develop morphology and synaptic inputs similar to standard Retzius cells (Loer, et al., 1987; Loer and Kristan, 1989; Loer and Kristan, 1989). In addition, cell lineage experiments in the leech *Theromyzon* (Gleizer and Stent, 1993) showed that, when a Rz cell that by cell lineage was located in a non-reproductive ganglion, but by a developmental anomaly sent its axons into a periphery that by cell lineage was reproductive (and that contained primordial reproductive ducts) the phenotype of the Rz cell switched to the reproductive pattern. These results suggest that the peripheral contacts of Retzius cells affect their morphology, their central morphology, and the synapses they form within the central nervous system.

The development of some motor neurons has also been shown to respond to peripheral contacts. Normally each motor neuron of a leech innervates specific regional muscle fibers in the body wall, and these peripheral contacts are established before central connections develop. Cutting the nerve roots on one side of a ganglion is followed by exuberant overgrowth of the processes of some motor neurons that

normally project to the periphery through those roots (Johnson, et al., 2000).

The goal of the present work was to alter the peripheral target of identified motor neurons to determine whether novel peripheral contacts would affect their morphology and connectivity. To force neurons to encounter inappropriate tissue as they exit the ganglion, we transplanted pieces of dorsal embryonic tissue into a ventral position adjacent to developing ganglia.

Methods

Animals

Adult medicinal leeches (*Hirudo sp.*) 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 days after egg deposition and were then held at 20–24°C in embryo water (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 0% (egg deposition) to 100% of embryonic development (Reynolds, et al., 1998). Surgeries were performed at 47.5-49% ED (Fig. 4.1A) 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 CaCl2, 1.5 MgCl2, 10 dextrose, 4.6 Tris maleate, and 5.4 Tris base, pH 7.4 and to which ethanol was added to produce a final concentration of 8% (v/v). Anesthetized embryos were placed in rectangular chambers custom-cut into Sylgard-lined plastic Petri dishes, 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.

To expose growing neurites to ectopic tissue, a square of ventral tissue was cut from the host embryo (Fig. 4.1), with the anterior boundary of the transplanted tissue located at the posterior margin of

the ganglion in the segment just anterior to the cut segment and the posterior boundary of the transplanted tissue located at the anterior boundary of the next posterior ganglion. The medial boundary of the cut was the 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 cuts were located between segments 8 and 13. Next, a square of tissue was cut from the donor embryo from the same segment into which it would be transplanted and using the same anterior and posterior boundaries as the host. In ectopic transplants, the medial boundary was the middle of the nephridium, and the lateral boundary was the edge of the germinal plate (Fig. 4.1B). In eutopic transplants, tissue was cut from the donor with the same boundaries as the tissue that was removed from the host (Fig. 4.1C). The donor tissue was then moved into the hole that had been created in the host embryo, taking care to maintain the anterior-posterior orientation. This tissue was covered with a small shard of cover slip to hold the transplanted tissue in place, and the recipient was allowed 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. Visual observation ensured that the transplant had healed properly.

Immunohistochemistry

Dissected embryos were fixed overnight in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 5°C. We then rinsed 6 x 15 minutes each with PBS (8g NaCl, 0.2g KCl, 1.44g of Na_2HPO_4, 0.24g of KH2PO4, in 1 liter, ph=7.4) before further processing. All axons in the tissue were labeled using a monoclonal antibody directed against acetylated tubulin (ACT; Sigma) and a protocol previously described (Jellies, et al., 1996). Briefly, the primary antibody 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 4° C. After 6 x 15 minute washes in PBS, the tissue was incubated in AlexaFluor 568-conjugated rabbit-anti mouse IgG at a dilution of 1:500 in 10% goat serum, and 1% Triton X-100 in PBS overnight at 4°C . The tissue was washed 24 hours later with 6 x 15 minutes washes in PBS, dehydrated though an ethanol series, cleared with methyl salicylate, and mounted in DePeX mounting medium (Electron Microscopy

Figure 4.1 Diagram of transplantation

A. Photograph of a leech embryo (dye was injected under the germinal plate to enhance contrast). The dorsal territory extends from the nephridopores to the edge of the germinal plate. Ventral territory is tissue between the nephridopores and the ventral midline. (photograph courtesy of Kathleen French). B. Diagram of an ectopic dorsal-to-ventral transplant. C. Diagram of a eutopic ventral-to-ventral transplant.
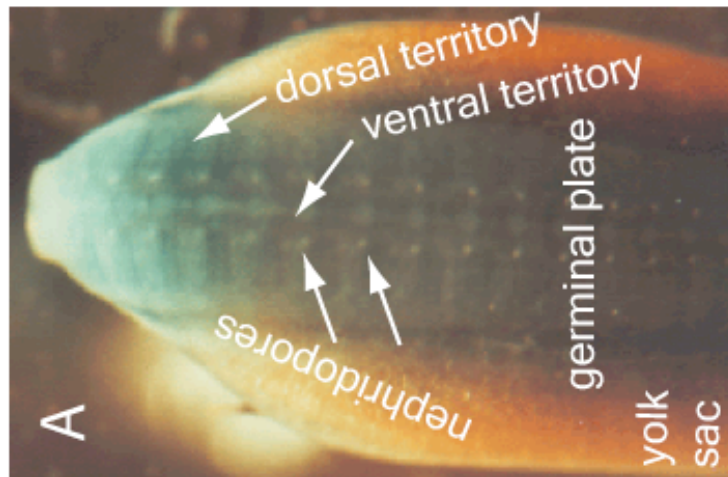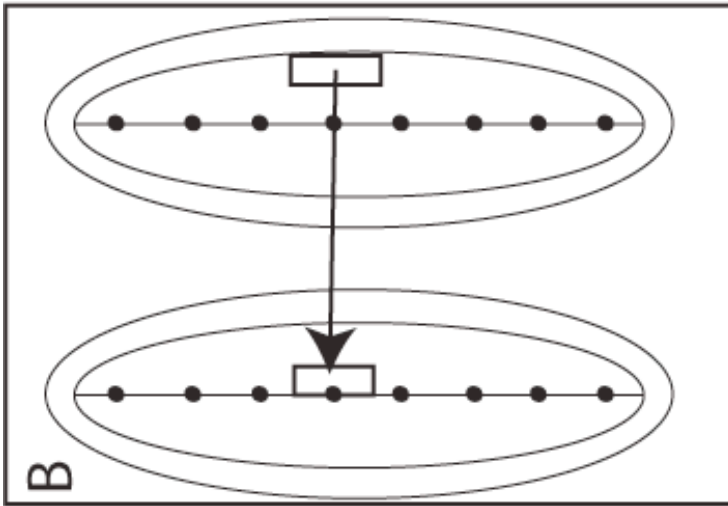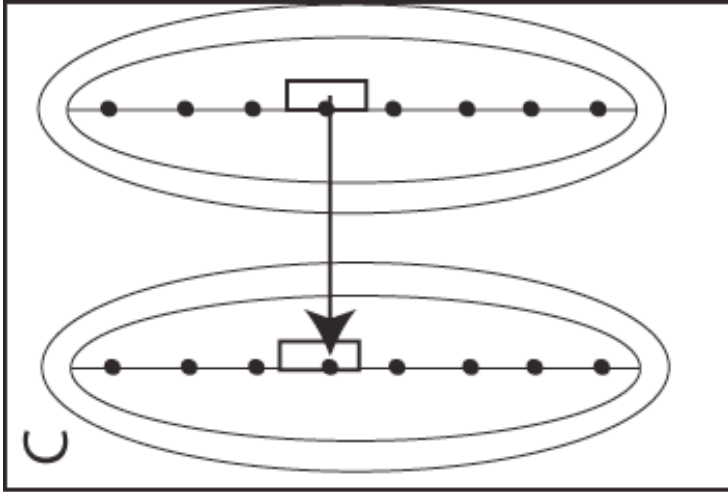
Sciences, Hatfield, PA, USA). These embryos were viewed using a Zeiss laser scanning confocal microscope using Bio-Rad software (Hercules, CA, USA).

Quantification of ACT staining

A threshold for ACT staining of axons in prepared whole mounts of the peripheral body wall was determined by calculating the average and standard deviation of the pixel intensity across the whole image (collapsed confocal Z stack) using ImageJ (http://rsbweb.nih.gov/ij/). A threshold value was defined as the average pixel intensity plus 1 standard deviation. To account for differences in animal size, we drew a square region of interest in the transplant tissue (or control tissue) 4 times the area of the ganglion adjacent to the transplant. We then applied the threshold to the region of interest (ROI) and determined the percentage of pixels within the ROI that were above threshold. We took this area to be the area of axons labeled by the anti-ACT antibody.

Intracellular injections for visualizing projection patterns

Embryos were anesthetized in 8% ethanol/leech saline, the nerve cord was exposed, and individual identified 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 Zeiss compound

microscope equipped with fluorescence optics. Dye was passed into

the cell using a sine-wave shaped depolarizing current protocol with a

1 second period and maximum current of +0.5 – +2 nA. The

fluorescence of the cell being filled was monitored periodically to

determine when to stop the injection. After 45 to 60 minutes, the living

preparation was imaged using a Zeiss laser-scanning confocal

microscope using Bio-Rad software (Hercules, CA, USA) to determine

the extent of that neuron's growth into the transplant region.

Inducing annulus erection

Each leech segment has 5 rings (annuli) visible on the surface of

the body, and tiny muscles called annulus erectors can transform the

smooth surface of the skin into sharp ridges (Muller, et al. 1981). The

annulus erector muscles are driven by a pair of motor neurons in each

ganglion; each of these neurons innervates the half of the body wall

contralateral to the soma, and a variety of sensory stimuli can lead to

activation of the annulus erector motor neurons (Stuart, 1970). We

induced the annuli to become erect by placing the embryos briefly

into ice-cold normal leech saline with 8% ethanol. This stimulus was

usually enough to cause annulus erection, but the dermis was

sometimes further stimulated by poking with a tungsten wire.

Semi-intact preparation for stimulating motor neurons and observing muscle contraction

After the embryos with transplants had reached the juvenile

stage, we made an incision to open along the lateral edge opposite

to the side where the transplant had healed. Most leech motor

neurons project across the ganglion and into the body wall

contralateral to the location of the soma (Muller, et al., 1981). The skin

of the leech was removed over the ganglion that was then pinned

dorsal-side up, which required a half-twist in the nerve roots. We

identified either cell DE-3 (a motor neuron that excites dorsal

longitudinal muscles in the body wall contralateral to the soma) or cell

VE-4 (a motor neuron that excites ventral longitudinal muscles in the

body wall ipsilateral to the soma) using a Wild dissecting microscope

outfitted with a video camera (C-Mounted Hitachi KP-M1

monochrome CCD camera (Image Labs International, Bozeman, MT)).

The images were captured at 10 Hz for 30 seconds using a Data

Translation frame grabber card (DT3155) controlled with the MATLAB

(The Mathworks, Natick, MA) Image Acquisition Toolbox on a PC

computer. On a different computer, intracellular recordings were

made using an Axoclamp-2A amplifier (Axon Instruments, Foster City,

CA) and Axograph 4.9 software (Axon Instruments, Union City, CA).

We applied 2 nA depolarizing current to the impaled motor neuron for

5 seconds and video-recorded the muscle contraction.

Intracellular injections to reveal dye-coupling

Dye coupling was assayed using a previously described

technique (Marin-Burgin, et al., 2006). Briefly, embryos were

anesthetized in 8% ethanol-leech saline, the nerve cord was exposed,

and individual neurons were impaled with sharp microelectrodes filled

with a combination of 2.5% (wt./vol.) Neurobiotin (Vector Laboratories,

Burlingame, CA, USA) and 2.5% lyseinated AlexaFluor 488 dextran

(10,000 MW) (Invitrogen) dissolved in de-ionized water. Dye was

passed with sine waves of depolarizing 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 ImmunoResearch, 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).

Statistics

t tests and ANOVA were used to evaluate statistical significance. All error bars are standard errors.

Results

Dorsal tissues retained their identity after transplantation

The goal of these experiments was to force ventral and dorsal motor neurons to encounter inappropriate tissue when their axons grew out of the ganglion. We were concerned, however, that following surgery the identity of the transplanted tissue might be induced by its new surrounding tissue to regulate; i.e. transplanted tissue from a dorsal location might take on ventral characteristics. Transplants were carried out in embryos that were 48% ED, and at this age, the dorsal and ventral embryonic tissues look the same – no body pigment or other morphological characteristics distinguish

between the two locations. To determine the fate of the transplants, we dissected and observed the animals18 days later, after they completed development and were juvenile leeches. By this time pigmentation had developed, and the color patterns confirmed that in a dorsal-to-ventral transplant, the transplanted tissue had retained its pre-transplant identity, as indicated by its characteristic pattern of pigmentation (Fig. 4.2 A and B). Additionally, the pigmentation revealed the boundaries of the transplanted tissue and indicated that the tissue had been smoothly integrated into the body wall.

In ventral-to-ventral, eutopic transplants, it was impossible to distinguish whether the transplanted tissue had been rejected by the host based on body pigmentation. In these animals, we could tell where the transplant was based on internal morphological characteristics. In a normal embryo, two lateral heart tubes carry blood the length of the body. In our ventral-to-ventral transplanted animals, we observed a discontinuity in the heart tubes at the point of the transplant. A portion of tube did develop in the transplant, but it failed to connect with the host's heart tube (Fig. 4.2 C and D).

Figure 4.2 Transplanted tissue retained its fate and was not rejected

A. Photograph of the dorsal aspect of a control juvenile leech. The pattern of pigment shown in this image is typical of juvenile *Hirudo sp*. The anterior of the animal is to the right. B. The ventral aspect of a juvenile leech that had received an ectopic dorsal-to-ventral transplant. The normal ventral light-green coloration is evident in most of the animal, but the area into which dorsal tissue was transplanted (white arrows) instead has a dark and stippled dorsal color pattern. Anterior is to the right, and the head of the animal has been bent to reveal the normal dorsal coloration. C. The ventral side of a control leech embryo ~ 80% ED. The lateral heart tube is evident as a continuous red zigzag stripe visible through the body wall. Anterior is to the right. D. Close-up view of the ventral aspect of a juvenile leech that had received a eutopic ventral-to-ventral transplant. The discontinuous heart tube (black arrows) reveals the anterior and posterior boundaries of the transplant.
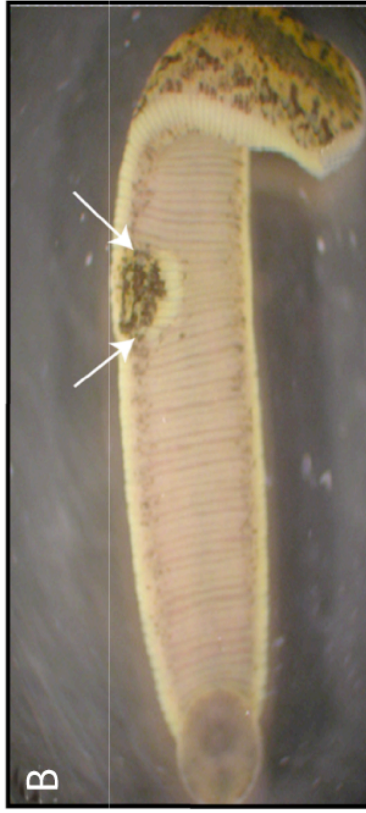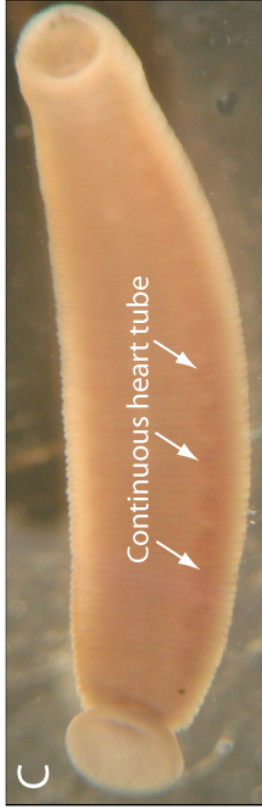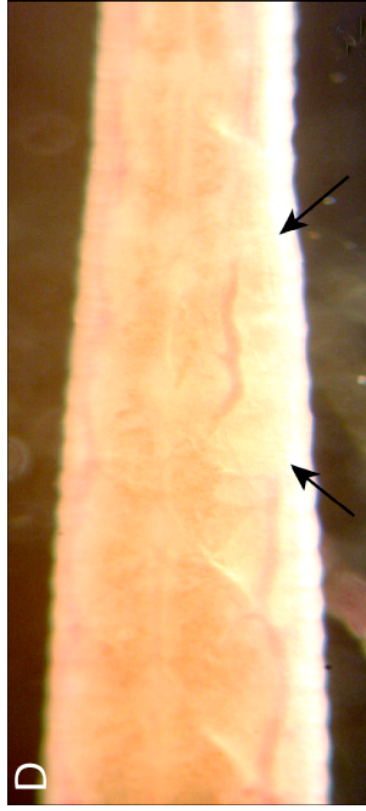
Continuous heart tube

Dorsal to Ventral Transplant

Ventral to Ventral Transplant

Transplanted tissues became innervated

To determine whether neurons from the adjacent ganglion grew into the transplanted tissue, we used an antibody to the cytoskeletal component, acetylated tubulin (ACT) (Jellies, et al., 1996) in whole, fixed embryos (Fig. 4.3 A). We quantified the amount of nerve growth into a region by calculating the percent area of ACT labeling within a region of interest normalized to 4 times the area of the nearest ganglion (see Methods for details). We found that all transplanted tissue (whether ectopic or eutopic) had the same amount of ACT label as intact controls and the intact areas of animals that received transplants (Fig. 4.3 B).

To determine whether motor neurons in the segment that received the transplanted tissue had arborized into the new tissue, we filled identified motor neurons with Alexa flour 488 (green) or 568 (red) and imaged the ganglion. We focused on two motor neurons that have been very well characterized: cell DE-3 and cell VE-4. We discovered that fixation and further tissue processing reduced the intensity of the fluorescence and made it impossible to visualize stained axons outside the ganglion, so in these experiments we imaged the filled cells in living, uncleared body wall.

In almost all cases the motor neurons projected out the ganglia

Figure 4.3 Neurons from the ganglion grew into transplanted tissue

A. Acetylated tubulin antibody stain of an intact juvenile leech. The ganglion is shown (arrow), and the connective nerves project anterior and posterior (up and down, respectively) from the ganglion. Peripheral nerves project laterally from either side of the ganglion and produce a symmetrical branching pattern in the body wall of the animal. B. Quantification of antibody staining. Measurements were made in the body wall directly adjacent to the ganglion. "Ventral region" and "dorsal region" show the percent of the ROI (as defined in Methods) that was scored as positive for acetylated tubulin in the ventral and dorsal regions of intact animals (n = 5, each). "Intact region of ectopic animal" was the percent area stained in an intact ventral region of a dorsal-to-ventral transplant animal (n = 4). "Ectopic" and "eutopic" indicate the percent area stained in the ROI within the area of the transplant (n = 5, each). There were no significant differences in the amount of acetylated tubulin staining in any of these regions.

B.

% area with positive ACT labeling
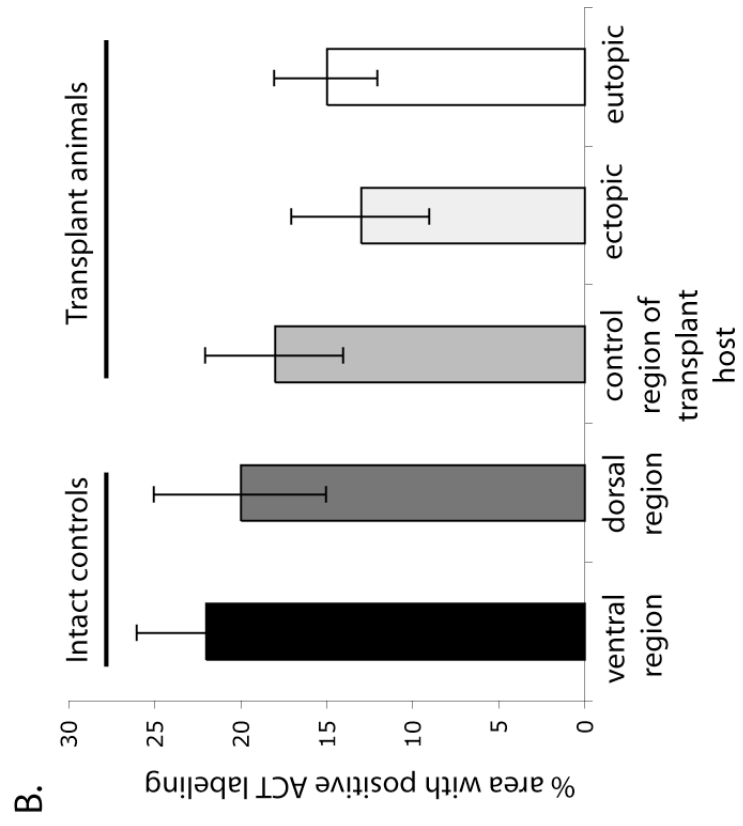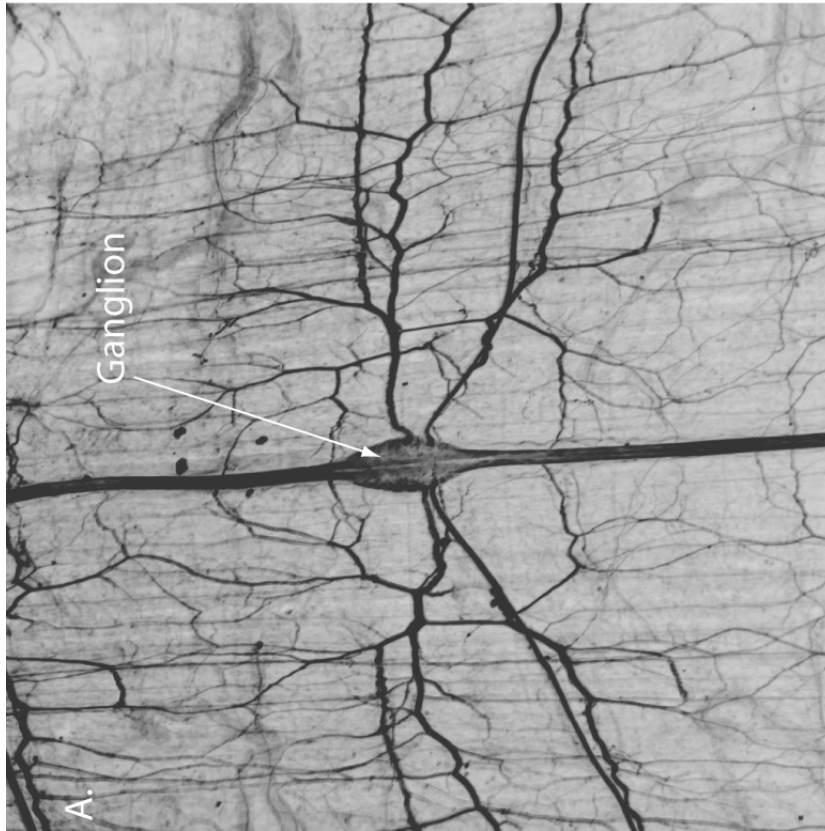
and into the adjacent body wall. However, when the motor neuron DE-3 was filled in animals that received eutopic transplants, it failed to project into the periphery and instead the processes remained within the CNS, projecting anteriorly in the connective as described in Johnson, et al. 2000 (Johnson, et al., 2000) (n = 4).

Transplanted tissue was functionally innervated

Anatomical evidence that axons grew into the transplanted tissue did not, however, guarantee that functional synapses formed there. To assay synapse formation we assayed for motor neuron function within the transplanted tissue. The annulus erector neurons (AE) are a pair of motor neurons in the posterior of the ganglion. Together, they are responsible for contracting the muscles that erect the annuli within their segment (Stuart, 1970). These muscles can be induced to contract by exposure to cold or by poking the skin with a sharp tungsten wire. In response to such stimulation, annuli within the transplant became erect (Fig. 4.4), indicating that the annulus erector motor neurons were functional and innervated the annulus erector muscles in the transplanted tissue.

To determine whether this activation of the annulus erector muscles was by activity in motor neurons in the ganglion adjacent to

the transplant, we dissected juvenile leeches with ectopic dorsal-to-ventral transplants and exposed the ganglion, leaving the nerve roots to that portion of the body wall intact. In these animals, after exiting the ganglion, the axon of cell DE-3 would have immediately encountered dorsal tissue, then a lateral edge, and finally the native dorsal tissue that it would normally innervate (Fig 4.5 A). We impaled cell DE-3 with a sharp microelectrode, stimulated it, and observed the body wall for contractions (Fig 4.5 B). When we stimulated cell DE-3, we observed that in 3 of 3 animals, only the transplanted dorsal tissue contracted -- the first dorsal tissue that the axon of cell DE-3 encountered (Fig 4.5 C). The native dorsal area did not contract, suggesting that when the axon of cell DE-3 entered the transplanted tissue, it stopped extending and began to branch, as cells DE-3 do during normal development when they reach their target region. When we stimulated the appropriate VE-4 in these preparations, there was no response in the body wall. In animals that received eutopic, ventral-to-ventral transplants, stimulating DE-3 had no effect on the body wall, indicating that cell DE-3 failed to innervate the longitudinal muscles in transplanted ventral body wall. Stimulating VE-4 in a ganglion adjacent to a eutopic transplant resulted in contraction of the transplanted ventral tissue.

Figure 4.4 Annuli in transplanted tissue responded to stimulation by becoming erect.

A. Appearance of relaxed annuli in normal body wall and in an ectopic, dorsal-to-ventral transplant. Along the edge of the body, the annuli are as smooth in transplanted tissue as in other portions of the body. B. The same animal after exposure to ice-cold saline; annuli become erect in normal body wall and in the transplant. C. Annuli became erect in eutopic, ventral-to-ventral transplants as well.
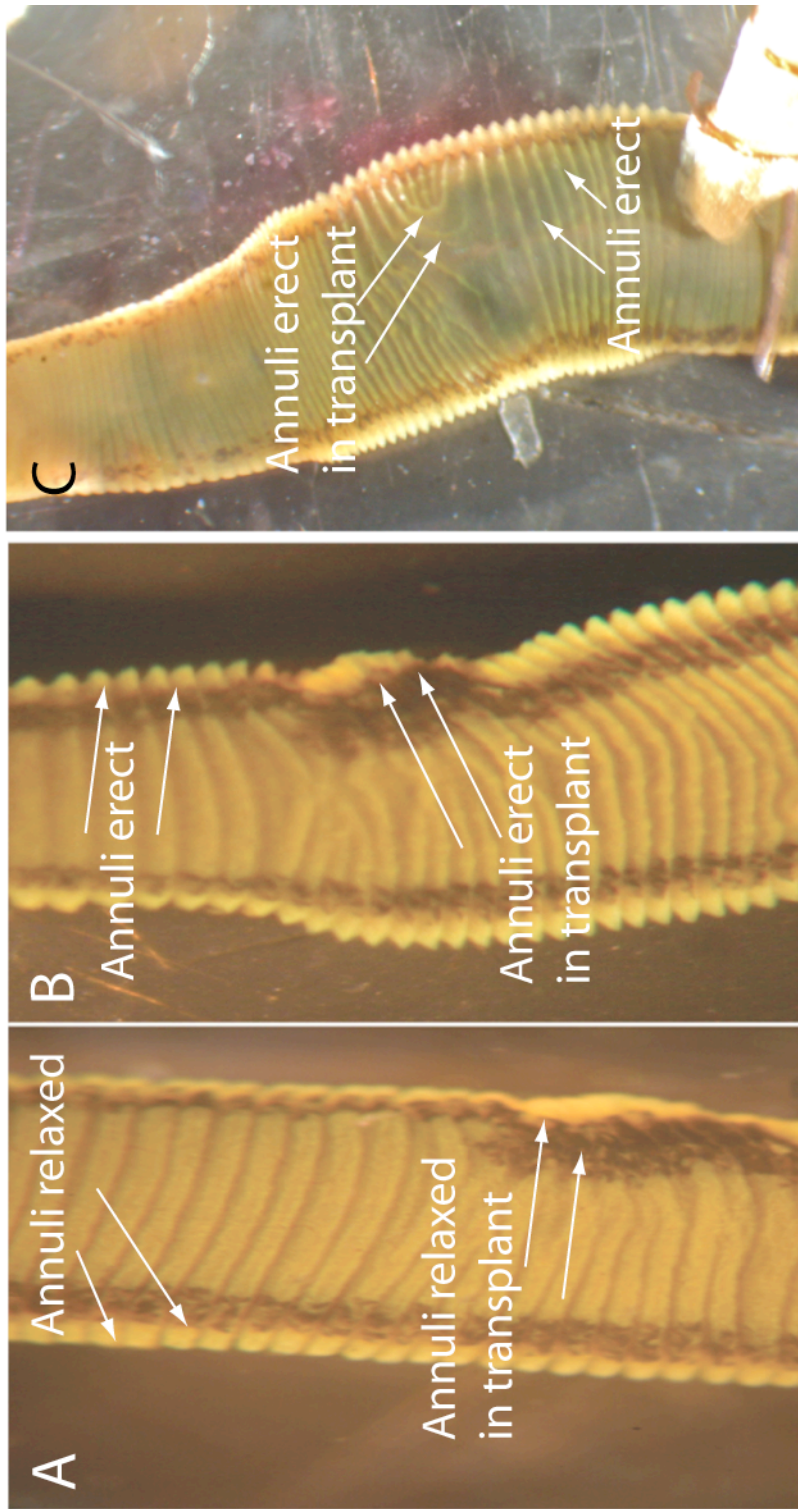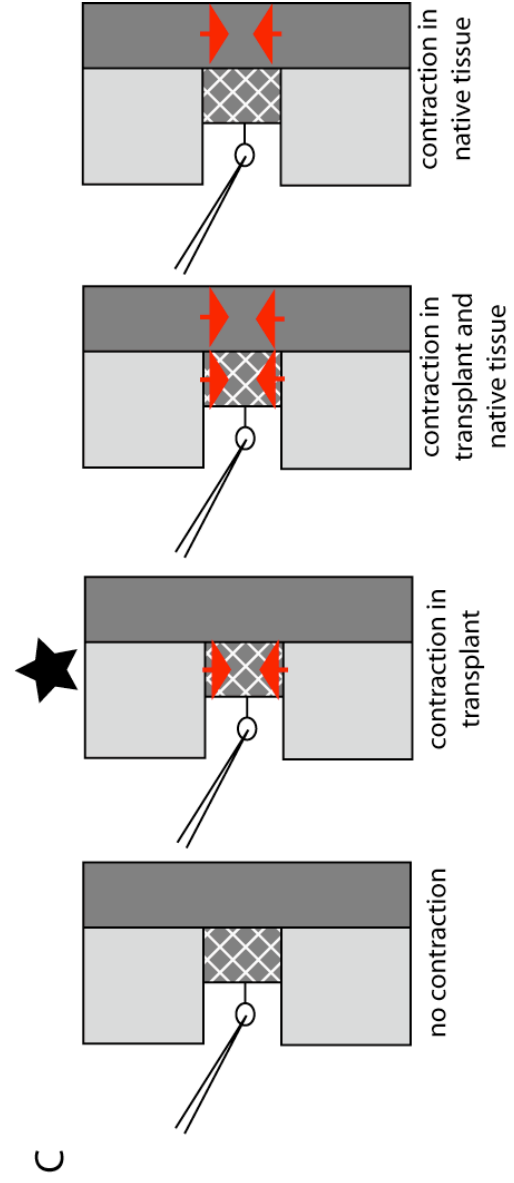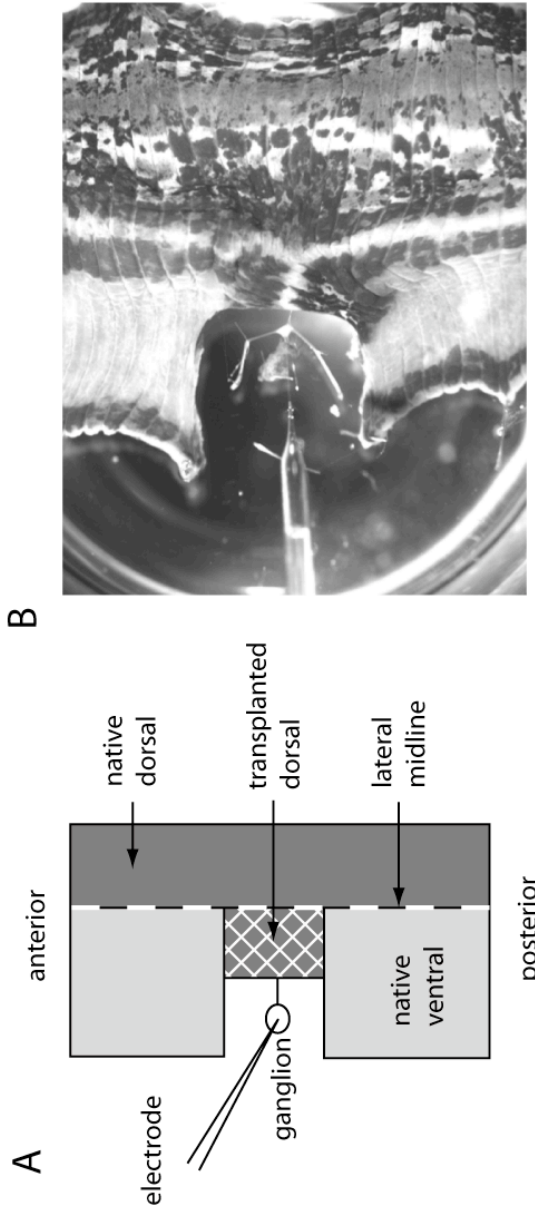
Figure 4.5 Excitation of cell DE-3 excited longitudinal muscles in transplanted dorsal body wall

A. Diagram of the semi-intact body wall preparation for studying contraction in response to motor neuron stimulation. Native tissue is in light grey, dorsal tissue is dark grey. The ganglion adjacent to the transplanted tissue is dissected to expose the motor neurons and in this example, dorsal longitudinal exciter DE-3 has been impaled with an intracellular electrode. B. Photograph of a dorsal-to-ventral semi-intact preparation. Arrows indicate the anterior and posterior boundaries of the darker, transplanted dorsal tissue next to the ganglion. C. Red arrows indicate contraction in four possible outcomes of exciting DE-3 (from left to right): no response, contraction in the transplanted dorsal tissue ending at the lateral edge, contraction in the transplanted dorsal and native dorsal body wall, or contraction in the native dorsal tissue only. The black star indicates that we saw contraction, but only in the transplanted dorsal tissue that was located immediately adjacent to the ganglion.

A
electrode
native dorsal
transplanted dorsal
lateral midline
ganglion
native ventral
anterior
posterior

B

C
no contraction
contraction in transplant
contraction in transplant and native tissue
contraction in native tissue

Central electrical synapses formed by cells DE-3 and VE-4 were unaltered by transplanted tissue

Previous work indicated that contact with peripheral targets plays an important role in determining some aspects of Retzius neurons phenotype, including peripheral and central morphology and central connectivity (Loer, et al., 1987). We performed ectopic and eutopic transplants in embryos at 45 - 50% ED and allowed the embryos to reach the juvenile stage. At that stage we exposed the dorsal surface of the ganglion in the segment that received the transplanted tissue and injected Neurobiotin into either cell DE-3 or cell VE-4. After the tissue was processed to reveal the electrically coupled cells, we acquired confocal images of each ganglion and counted the number of coupled cells. We saw that the number of cells dye-coupled to cell DE-3 and VE-4 remained the same as in normal segments (Fig. 4.6). We are unable to conclude whether cell DE-3 and cell VE-4 were coupled to precisely the same neurons following transplants, but the dye-coupled cells did appear in similar locations from preparation to preparation.

Figure 4.6 Electrical synapses made by cell DE-3 and cell VE-4 were unaltered by exposure to inappropriate peripheral tissue.

Number of cells dye-coupled to cell DE-3 and cell VE-4 in ganglia adjacent to either ectopic or eutopic transplants. Transplants were carried out at 45 - 48% ED, and dye-coupling assays were done after the embryos reached the juvenile stage. Following transplants, the number of dye-coupled cells was not significantly different from unmanipulated animals (ANOVA).

A   Neurobiotin in DE3

B   Neurobiotin in DE4
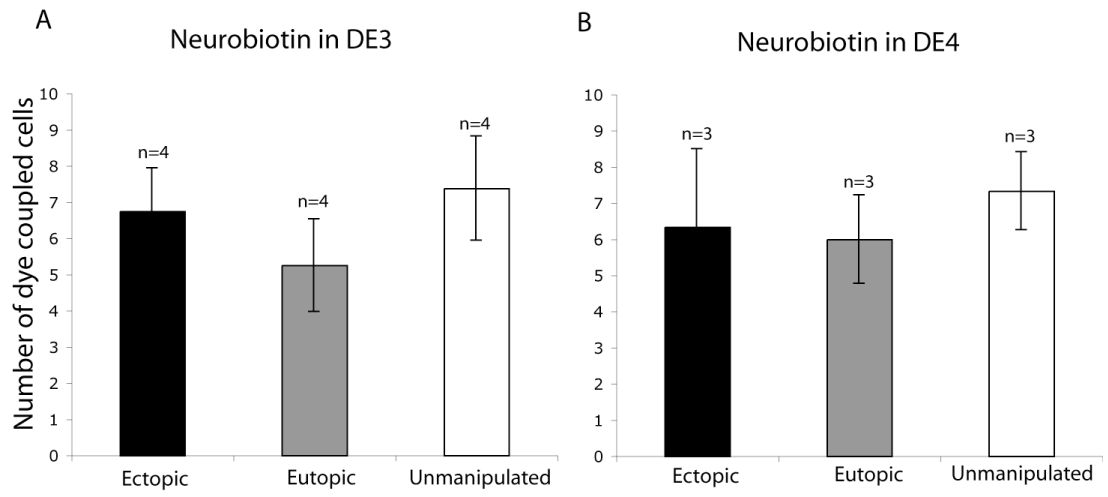
Discussion

In our experiments we found that tissue transplants retain their original fate when performed in 48% ED developing leeches. Nerves from adjacent ganglia grew into the transplanted tissue, and the muscles in the transplanted pieces of body wall became appropriately and functionally innervated. That is, cell DE-3, a dorsal longitudinal exciter synapses onto longitudinal muscles; we saw no indication of synapses onto other types of fibers, such as circular muscles. in the transplanted region. Individual, identified motor neurons that innervated transplanted tissue formed electrical synapses with the appropriate number of neurons with their ganglia.

In segments that received ectopic transplants of dorsal body wall, cell DE-3 synapsed onto the longitudinal muscles of the transplanted dorsal tissue, but not past the lateral edge and into the native dorsal tissue. Following eutopic transplants, cell VE-4 sent axons into the transplanted area, but cell DE-3 sent long processes into the anterior connective nerve, which never occurs in normal development. This observation is not surprising based on previous results (Johnson, et al., 2000), in which cutting the nerve roots on one side of a single ganglion in embryos at about 50% ED, isolating the

ganglia from the periphery, caused cell DE-3 to branch wildly within the ganglion.

Our findings raise several questions. Why does the axon of cell DE-3 grow into a dorsal-to-ventral transplant, yet not into a ventral-to-ventral transplant – tissue that it would normally encounter? One possibility is that because the pressure-sensitive mechanosensory P cell helps to pioneer peripheral nerves (Kuwada and Kramer, 1983; Kuwada, 1985) when this pathway is cut during the transplant procedure, a cell DE-3 axon may be able to navigate through dorsal body wall, but cannot find its way through ventral territory or across the lateral edge. It is interesting to note that when the roots were cut with no subsequent transplantation of body wall, the neurons that did send axons out of the ganglion included the ventral motor neurons (Johnson, et al., 2000).

Why does cell DE-3 form functional synapses within the transplanted dorsal tissue, but fail to cross the lateral edge and synapse in the native dorsal tissue? There is evidence that peripheral targets are capable of producing molecular 'stop' signals that induce the formation of neuromuscular junctions (Campagna, et al., 1995). When cell DE-3 immediately encounters dorsal tissue in a dorsal-to-ventral transplant perhaps it recognizes the molecular 'stop' signal of

its designated target and changes into synaptogenesis mode. We observed that cell DE-3 sent an axon into ectopic transplanted tissue and formed excitatory synapses onto longitudinal muscle, but the contraction caused by stimulating this motor neuron did not extend beyond the transplant area. We conclude that the axon of cell DE-3 failed to innervate the native dorsal tissue adjacent to the transplant. One explanation for this observation depends on the nature of the transplanted tissue. The dorsal transplant was collected at the lateral edge of the germinal plate, i.e., with the eventual dorsal midline tissue forming the lateral edge of the transplanted tissue) (see Figure 4.1). Leech motor neurons typically do not cross the midline in the periphery, so there may well be a 'stop' signal at the dorsal midline, which in our transplants would have been located at the lateral edge of the body wall, proximal to the native dorsal tissue. Such 'stop' signals have been reported from other axon guidance research (Reeber, et al., 2008; Yokoyama, et al., 2001).

In contrast to the behavior of cell DE-3, cell VE-4 had no problem growing into the ventral tissue of a eutopic transplant or, indeed, into transplanted dorsal tissue. Why should it behave so differently? We saw that VE-4 extends its processes into the dorsal transplants, but did not make synapses there. Perhaps these cells,

which ordinarily have no need of navigating through "foreign" territory before reaching their peripheral target field, do not require the aid of a pioneer neuron to set out the pathway and instead grow into adjacent tissue regardless of what it is. However, they may still require a ventral tissue-specific cue to begin forming synapses with muscle fibers. All of these remain active questions that we are pursuing.

Acknowledgements

Chapter 5: Concluding Remarks

The chapters contained within this thesis employed a variety of strategies for examining how the neurons of the leech nervous system form appropriate synapses centrally and peripherally. The developmental consequences of appropriate synapse formation and connectivity are not a problem that only the medicinal leech has overcome. Therefore, I propose that the findings of these experiments may apply broadly to a variety of species.

Chapter 2 describes evidence supporting a long-standing hypothesis that electrical synapses help to establish the chemical synaptic connectivity of the mature nervous system. Using a highly specific RNA interference technique in single pressure-mechanosensory P cells, we have shown that chemical synaptic connections fail to form between normally strong synaptic partners when electrical synapse function is decreased in developing leech embryos. This deficit is still evident in the behavior of the animals months later, telling us that the synaptic disruption includes most or all of the connections that a P cell should make. Further, we showed that the P cells received sensory input normally, indicating that the

behavioral consequence was purely a result of aberrant P cell chemical synaptic connectivity.

Chapter 3 describes a technique that will allow us to probe the development of synaptic connectivity among motor neurons. The somata of these cells are located on the dorsal surface of each ganglion and thus require that the embryos be held in organ culture and nearly motionless to study the development of these neurons over time. By embedding the dissected embryos in agarose and maintaining them in a culture medium, we have shown that the RNA interference method used in Chapter 2 also works to block innexin-1 based electrical synapses in motor neurons. We will use the combination of RNAi and the ability to study motor neurons long-term to address the development and nature of rectifying electrical synapses. Using these techniques, we will be able to determine whether the rectifying property of these synapses is due to the expression of different innexins in the two connected neurons.

In Chapter 4, we reported that despite being forced to interact with inappropriate peripheral tissue, motor neurons DE-3 and VE-4 seem to maintain important features of their cellular identities: they project into peripheral tissues, whether or not the tissue is their normal target, but they form functional synapses only with appropriate muscle

fibers, and they maintain the normal complement of dye-coupled cells within the ganglion. The pattern of axon extension and synaptogenesis suggests that the embryonic leech may be a tractable organism for studying some of the same molecular cues for axis formation and axon guidance, which could then be applied to vertebrate models. As in other sub-disciplines of neurobiology, such as the neuronal basis of behavior, these phenomena may benefit from the relative simplicity of the nervous system of the leech.

References

Akaaboune, M., Culican, S.M., Turney, S.G., & Lichtman, J.W., Rapid and reversible effects of activity on acetylcholine receptor density at the neuromuscular junction in vivo. *Science* 286 (5439), 503-507 (1999).

Angevine, J.B., Jr. & Sidman, R.L., Autoradiographic study of cell migration during histogenesis of cerebral cortex in the mouse. *Nature* 192, 766-768 (1961).

Baca, S.M., Marin-Burgin, A., Wagenaar, D.A., & Kristan, W.B., Jr., Widespread inhibition proportional to excitation controls the gain of a leech behavioral circuit. *Neuron* 57 (2), 276-289 (2008).

Baca, S.M., Thomson, E.E., & Kristan, W.B., Location and intensity discrimination in the leech local bending response quantified using optic flow and principal components analysis. *J. Neurophysiol.* In Press (2005).

Baca, S.M., Thomson, E.E., & Kristan, W.B., Jr., Location and intensity discrimination in the leech local bend response quantified using optic flow and principal components analysis. *J Neurophysiol* 93 (6), 3560-3572 (2005).

Bani-Yaghoub, M., Bechberger, J.F., Underhill, T.M., & Naus, C.C., The effects of gap junction blockage on neuronal differentiation of human NTera2/clone D1 cells. *Experimental neurology* 156 (1), 16-32 (1999).

Bani-Yaghoub, M., Underhill, T.M., & Naus, C.C., Gap junction blockage interferes with neuronal and astroglial differentiation of mouse P19 embryonal carcinoma cells. *Developmental genetics* 24 (1-2), 69-81 (1999).

Baptista, C.A., Gershon, T.R., & Macagno, E.R., Peripheral organs control central neurogenesis in the leech. 346 (6287), 855-858 (1990).

Bennett, M.V., Physiology of electrotonic junctions. *Ann NY Acad Sci* 137, 509-539 (1966).

Bennett, M.V., Aljure, E., Nakajima, Y., & Pappas, G.D., Electrotonic junctions between teleost spinal neurons: electrophysiology and ultrastructure. *Science* 141, 262-264 (1963).

Bennett, M.V.L., Electrical synapses, a personal perspective (or history). *Brain Res. Rev* 32 (1 Special Issue SI), 16-28 (2000).

Bennett, M.V.L., Seeing is relieving: electrical synapses between visualized neurons. *Nat. Neurosci* 3 (1), 7-9 (2000).

Bentley, D. & Caudy, M., Pioneer axons lose directed growth after selective killing of guidepost cells. *Nature* 304 (5921), 62-65 (1983).

Bonnot, A.S., Whelan, P.J., Mentis, G.Z., & O'Donovan, M.J., Spatiotemporal pattern of motoneuron activation in the rostral lumbar and the sacral segments during locomotor-like activity in the neonatal mouse spinal cord. *J. Neurosci* 22 (3), NIL_1-NIL_6 (2002).

Borodinsky, L.N. *et al.*, Activity-dependent homeostatic specification of transmitter expression in embryonic neurons. *Nature* 429 (6991), 523-530 (2004).

Borrell, V. & Callaway, E.M., Reorganization of exuberant axonal arbors contributes to the development of laminar specificity in ferret visual cortex. *J. Neurosci* 22 (15), 6682-6695 (2002).

Brummelkamp, T.R., Bernards, R., & Agami, R., A system for stable expression of short interfering RNAs in mammalian cells. *Science* 296 (5567), 550-553 (2002).

Bureau, I., Shepherd, G.M.G., & Svoboda, K., Precise development of functional and anatomical columns in the neocortex. *Neuron* 42 (5), 789-801 (2004).

Campagna, J.A., Ruegg, M.A., & Bixby, J.L., Agrin is a differentiation-inducing "stop signal" for motoneurons in vitro. *Neuron* 15 (6), 1365-1374 (1995).

Clarke E, a.O.M.C., *The Human Brain and Spinal Cord: A Historical Study*, 2nd ed. (1996).

Cohen-Cory, S., The developing synapse: construction and modulation of synaptic structures and circuits. *Science* 298 (5594), 770-776 (2002).

Crespel, F., Mariani, J., & Delhaye-Bouchaud, N., Evidence for a multiple innervation of purkinje cells by climbing fibers in the immature rat cerebellum. *Journal of Neurobiology* 7 (6), 567-578 (1976).

Curtin, K.D., Zhang, Z., & Wyman, R.J., Gap junction proteins expressed during development are required for adult neural function in the Drosophila optic lamina. *J Neurosci* 22 (16), 7088-7096 (2002).

de Rivero Vaccari, J.C., Corriveau, R.A., & Belousov, A.B., Gap junctions are required for NMDA receptor dependent cell death in developing neurons. *J Neurophysiol* 98 (5), 2878-2886 (2007).

Deans, M.R., Gibson, J.R., Sellitto, C., Connors, B.W., & Paul, D.L., Synchronous activity of inhibitory networks in neocortex requires electrical synapses containing Connexin36. *Neuron* 31 (3), 477-485 (2001).

Debski, E.A. & Cline, H.T., Activity-dependent mapping in the retinotectal projection. *Curr. Opin. Neurobiol* 12 (1), 93-99 (2002).

Dietzel, I.D. & Gottmann, K., Development of dopamine-containing neurons and dopamine uptake in embryos of Hirudo medicinalis. *Dev Biol* 128 (2), 277-283 (1988).

Drapeau, P., Catarsi, S., & Merz, D.C., Signalling synapse formation between identified neurons. *Journal of Physiology, Paris* 89 (3), 115-123 (1995).

Drapeau, P. *et al.*, Development of the locomotor network in zebrafish. *Prog. Neurobiol* 68 (2), 85-111 (2002).

Dykes, I.M., Freeman, F.M., Bacon, J.P., & Davies, J.A., Molecular basis of gap junctional communication in the CNS of the leech Hirudo medicinalis. *J. Neurosci* 24 (4), 886-894 (2004).

Dykes, I.M. & Macagno, E.R., Molecular characterization and embryonic expression of innexins in the leech Hirudo medicinalis. *Dev Genes Evol* 216 (4), 185-197 (2006).

Elias, L.A., Wang, D.D., & Kriegstein, A.R., Gap junction adhesion is necessary for radial migration in the neocortex. *Nature* 448 (7156), 901-907 (2007).

Elliott, T.R., The action of adrenalin. *J Physiol* 32 (5-6), 401-467 (1905).

Fan, R.J., Marin-Burgin, A., French, K.A., & Otto Friesen, W., A dye mixture (Neurobiotin and Alexa 488) reveals extensive dye-coupling among neurons in leeches; physiology confirms the connections. *Journal of comparative physiology* 191 (12), 1157-1171 (2005).

Feller, M.B., Wellis, D.P., Stellwagen, D., Werblin, F.S., & Shatz, C.J., Requirement for cholinergic synaptic transmission in the propagation of spontaneous retinal waves. *Science* 272 (5265), 1182-1187 (1996).

Fire, A. *et al.*, Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* 391 (6669), 806-811 (1998).

Fischbach, G.D., Synapse formation between dissociated nerve and muscle cells in low density cell cultures. *Developmental Biology* 28 (2), 407-429 (1972).

Flavell, R.B., Inactivation of gene expression in plants as a consequence of specific sequence duplication. *Proc Natl Acad Sci U S A* 91 (9), 3490-3496 (1994).

Fortier, E. & Belote, J.M., Temperature-dependent gene silencing by an expressed inverted repeat in Drosophila. *Genesis* 26 (4), 240-244 (2000).

Fox, M.A. *et al.*, Distinct target-derived signals organize formation, maturation, and maintenance of motor nerve terminals. *Cell* 129 (1), 179-193 (2007).

Frank, E. & Westerfield, M., Development of sensory motor synapses in the spinal cord of the frog rana-catesbeiana. *Journal of Physiology* 343, 593-610 (1983).

Fredette, B.J. & Landmesser, L.T., A reevaluation of the role of innervation in primary and secondary myogenesis in developing chick muscle. *Dev Biol* 143 (1), 19-35 (1991).

Furshpan, E.J. & Potter, D.D., Mechanism of nerve-impulse transmission at a crayfish synapse. *Nature* 180 (4581), 342-343 (1957).

Furshpan, E.J. & Potter, D.D., Transmission at the giant motor synapses of the crayfish. *Journal of Physiology* 145, 289-325 (1959).

Gabel, C.V., Antoine, F., Chuang, C.F., Samuel, A.D., & Chang, C., Distinct cellular and molecular mechanisms mediate initial axon development and adult-stage axon regeneration in C. elegans. *Development* 135 (6), 1129-1136 (2008).

Galarreta, W. & Hestrin, S., A network of fast-spiking cells in the neocortex connected by electrical synapses. *Nature* 402 (6757), 72-75 (1999).

Gibson, J.R., Beierlein, M., & Connors, B.W., Two networks of electrically coupled inhibitory neurons in neocortex. *Nature* 402 (6757), 75-79 (1999).

Gleizer, L. & Stent, G.S., Developmental origin of segmental identity in the leech mesoderm. *Development* 117 (1), 177-189 (1993).

Godfrey, E.W., Nitkin, R.M., Wallace, B.G., Rubin, L.L., & McMahan, U.J., Components of Torpedo electric organ and muscle that cause aggregation of acetylcholine receptors on cultured muscle cells. *J Cell Biol* 99 (2), 615-627 (1984).

Gu, X.N. & Spitzer, N.C., Breaking the code - regulation of neuronal differentiation by spontaneous calcium transients. *Developmental Neuroscience* 19 (1), 33-41 (1997).

Hagiwara, S. & Morita, H., Electrotonic transmission between two nerve cells in leech ganglion. *J Neurophysiol* 25, 721-731 (1962).

Hamburger, V., Some aspects of the embryology of behavior. *The Quarterly Review of Biology* 38 (4), 342-365 (1963).

Harrison, R.G., *Organization and Development of the Embryo*. (Garland Publishing, New York and London, 1988).

Hewapathirane, D.S., Dunfield, D., Yen, W., Chen, S., & Haas, K., In vivo imaging of seizure activity in a novel developmental seizure model. *Experimental neurology* 211 (2), 480-488 (2008).

Holland, R.L. & Brown, M.C., Postsynaptic transmission block can cause terminal sprouting of a motor nerve. *Science* 207 (4431), 649-651 (1980).

Hubel, D.H. & Wiesel, T.N., Effects of Monocular Deprivation in Kittens. *Naunyn Schmiedebergs Arch Exp Pathol Pharmakol* 248, 492-497 (1964).

Hunt, R.K. & Jacobson, M., Development and stability of postional information in Xenopus retinal ganglion cells. *Proc Natl Acad Sci U S A* 69 (4), 780-783 (1972).

Jasanoff, A. & Sun, P.Z., In vivo magnetic resonance microscopy of brain structure in unanesthetized flies. *J Magn Reson* 158 (1-2), 79-85 (2002).

Javaherian, A. & Cline, H.T., Coordinated motor neuron axon growth and neuromuscular synaptogenesis are promoted by CPG15 in vivo. *Neuron* 45 (4), 505-512 (2005).

Jellies, J. & Johansen, J., Multiple strategies for directed growth cone extension and navigation of peripheral neurons. *J Neurobiol* 27 (3), 310-325 (1995).

Jellies, J., Kopp, D.M., Johansen, K.M., & Johansen, J., Initial formation and secondary condensation of nerve pathways in the medicinal leech. *J Comp Neurol* 373 (1), 1-10 (1996).

Jellies, J., Loer, C.M., & Kristan, W.B., Jr., Morphological changes in leech Retzius neurons after target contact during embryogenesis. *J Neurosci* 7 (9), 2618-2629 (1987).

Johnson, L.A., Kristan, W.B., Jr., Jellies, J., & French, K.A., Disruption of peripheral target contact influences the development of identified central dendritic branches in a leech motor neuron in vivo. *J. Neurobiol* 43 (4), 365-378 (2000).

Kandler, K. & Katz, L.C., Neuronal coupling and uncoupling in the developing nervous system. *Curr. Opin. Neurobiol* 5, 98-105 (1995).

Katz, L.C., Specificity in the development of vertical connections in cat striate cortex. *European Journal of Neuroscience* 3 (1), 1-10 (1991).

Katz, L.C. & Shatz, C.J., Synaptic activity and the construction of cortical circuits. *Science* 274 (5290), 1133-1138 (1996).

Kiehn, O. & Tresch, M.C., Gap junctions and motor behavior. *Trends Neurosci* 25 (2), 108-115 (2002).

Kirsch, J., Malosio, M.L., Wolters, I., & Betz, H., Distribution of gephyrin transcripts in the adult and developing rat brain. *Eur J Neurosci* 5 (9), 1109-1117 (1993).

Kirsch, J., Wolters, I., Triller, A., & Betz, H., Gephyrin antisense oligonucleotides prevent glycine receptor clustering in spinal neurons. *Nature* 366 (6457), 745-748 (1993).

Kristan, W.B., Jr., Calabrese, R.L., & Friesen, W.O., Neuronal control of leech behavior. *Prog Neurobiol* 76 (5), 279-327 (2005).

Kristan, W.B., Jr., Eisenhart, F.J., Johnson, L.A., & French, K.A., Development of neuronal circuits and behaviors in the medicinal leech. *Brain Research Bulletin* 53 (5), 561-570 (2000).

Kumar, N.M. & Gilula, N.B., The gap junction communication channel. *Cell* 84 (3), 381-388 (1996).

Kuwada, J. & Kramer, A., Embryonic development of the leech nervous system: primary axon outgrowth of identified neurons. *J. Neurosci.* 3 (10), 2098-2111 (1983).

Kuwada, J.Y., Pioneering and pathfinding by an identified neuron in the embryonic leech. *J Embryol Exp Morphol* 86, 155-167 (1985).

Kuwada, J.Y. & Kramer, A.P., Embryonic development of the leech nervous system: primary axon outgrowth of identified neurons. *J Neurosci* 3 (10), 2098-2111 (1983).

Landmesser, L.T., The acquisition of motoneuron subtype identity and motor circuit formation. *Int J Dev Neurosci* 19 (2), 175-182 (2001).

Langenberg, T., Brand, M., & Cooper, M.S., Imaging brain development and organogenesis in zebrafish using immobilized embryonic explants. *Dev Dyn* 228 (3), 464-474 (2003).

Lent, C.M. & Frazer, B.M., Connectivity of the monoamine-containing neurones in central nervous system of leech. *Nature* 266 (5605), 844-847 (1977).

Levin, M., Isolation and community: A review of the role of gap-junctional communication in embryonic patterning. *Journal of Membrane Biology* 185 (3), 177-192 (2002).

Lewis, J.E. & Kristan, W.B., Jr., A neuronal network for computing population vectors in the leech. *Nature* 391 (6662), 76-79 (1998).

Lewis, J.E. & Kristan, W.B., Jr., Representation of Touch Location by a Population of Leech Sensory Neurons. *J Neurophysiol* 80 (5), 2584-2592 (1998).

Lewis, J.E. & Kristan, W.B.J., Quantitative Analysis of a Directed Behavior in the Medicinal Leech: Implications for Organizing Motor Output. *J. Neurosci* 18, 1571-1582. (1998).

Lichtman, J.W. & Colman, H., Synapse elimination and indelible memory. *Neuron* 25 (2), 269-278 (2000).

Lockery, S.R. & Kristan, W.B.J., Two forms of sensitization of the local bending reflex of the medicinal leech. *J. Comp. Physiol. A* 168 (2), 165-177 (1991).

Loeb, J.A., Hmadcha, A., Fischbach, G.D., Land, S.J., & Zakarian, V.L., Neuregulin expression at neuromuscular synapses is modulated

by synaptic activity and neurotrophic factors. *J Neurosci* 22 (6), 2206-2214 (2002).

Loer, C.M., Jellies, J., & Kristan, W.B., Jr., Segment-specific morphogenesis of leech Retzius neurons requires particular peripheral targets. *J Neurosci* 7 (9), 2630-2638 (1987).

Loer, C.M. & Kristan, W.B.J., Central synaptic inputs to identified leech neurons determined by peripheral targets. *Science* 7, 64-66 (1989).

Loer, C.M. & Kristan, W.B.J., Peripheral target choice by homologous neurons during embryogenesis of the medicinal leech ii. innervation of ectopic reproductive tissue by nonreproductive retzius cells. *J. Neurosci* 9 (2), 528-538 (1989).

Long, M.A., Landisman, C.E., & Connors, B.W., Small clusters of electrically coupled neurons generate synchronous rhythms in the thalamic reticular nucleus. *J. Neurosci* 24 (2), 341-349 (2004).

Lopresti, V., Macagno, E.R., & Levinthal, C., Structure and development of neuronal connections in isogenic organisms: transient gap junctions between growing optic axons and lamina neuroblasts. *Proc Natl Acad Sci U S A* 71 (4), 1098-1102 (1974).

Macagno, E.R., Number and distribution of neurons in leech segmental ganglia. *J Comp Neurol* 190 (2), 283-302 (1980).

Marder, E. & Calabrese, R.L., Principles of rhythmic motor pattern generation. *Physiological Reviews* 76 (3), 687-717 (1996).

Marin-Burgin, A., Eisenhart, F.J., Baca, M.S., Kristan, W.B., Jr., & French, K.A., Sequential development of electrical and chemical synaptic connections generates a specific behavioral circuit in the leech. *J. Neurosci.* 25 (10), 2478-2489 (2005).

Marin-Burgin, A., Eisenhart, F.J., Baca, S.M., Kristan, W.B., Jr., & French, K.A., Sequential development of electrical and chemical synaptic connections generates a specific behavioral circuit in the leech. *J Neurosci* 25 (10), 2478-2489 (2005).

Marin-Burgin, A., Eisenhart, F.J., Kristan, W.B., Jr., & French, K.A., Embryonic electrical connections appear to pre-figure a behavioral circuit in the leech CNS. *Journal of comparative physiology* 192 (2), 123-133 (2006).

Mason, A. & Kristan, W.B.J., Neuronal excitation, inhibition, and modulation of leech longitudinal muscle. *Journal of Comparative Physiology A Sensory Neural & Behavioral Physiology* 146, 527-536. (1982).

Milner, L.D. & Landmesser, L.T., Cholinergic and GABAergic inputs drive patterned spontaneous motoneuron activity before target contact. *J. Neurosci* 19 (8), 3007-3022 (1999).

Muller, K.J., Nicholls, J.G., & Stent, G.S., *Neurobiology of the leech*. (Cold Spring Harbor Laboratory, 1981).

Napoli, S., Pastori, C., Magistri, M., Carbone, G.M., & Catapano, C.V., Promoter-specific transcriptional interference and c-myc gene silencing by siRNAs in human cells. *EMBO J* 28 (12), 1708-1719 (2009).

Nicholls, J.G. & Baylor, D.A., Specific modalities and receptive fields of sensory neurons in CNS of the leech. *J. Neurophysiol* 31, 740-756 (1968).

Nose, A., Van Vactor, D., Auld, V., & Goodman, C.S., Development of neuromuscular specificity in Drosophila. *Cold Spring Harb Symp Quant Biol* 57, 441-449 (1992).

O'Donovan, M.J. & Chub, N., Population behavior and self-organization in the genesis of spontaneous rhythmic activity by developing spinal networks. *Seminars in Cell & Developmental Biology* 8 (1), 21-28 (1997).

O'Lague, P.H., Furshpan, E.J., & Potter, D.D., Studies on rat sympathetic neurons developing in cell culture. II. Synaptic mechanisms. *Dev Biol* 67 (2), 404-423 (1978).

Oakley, R.A. *et al.*, Neurotrophin-3 promotes the differentiation of muscle spindle afferents in the absence of peripheral targets. *J Neurosci* 17 (11), 4262-4274 (1997).

Ort, C.A., Kristan, W.B., Jr., & Stent, G.S., Neuronal control of swimming in the medicinal leech part 2 identification and connections of motor neurons. *Journal of Comparative Physiology A Sensory Neural & Behavioral Physiology* 94 (2), 121-154 (1974).

Peinado, A., Yuste, R., & Katz, L.C., Gap junctional communication and the development of local circuits in neocortex. *Cerebral Cortex* 3 (5), 488-498 (1993).

Penn, A.A., Wong, R.O.L., & Shatz, C.J., Neuronal coupling in the developing mammalian retina. *J. Neurosci* 14 (6), 3805-3815 (1994).

Phelan, P., Bacon, J.P., A. Davies, J., Stebbings, L.A., & Todman, M.G., Innexins: a family of invertebrate gap-junction proteins. *Trends in Genetics* 14 (9), 348-349 (1998).

Phelan, P. & Starich, T.A., Innexins get into the gap. *Bioessays* 23 (5), 388-396 (2001).

Reeber, S.L. *et al.*, Manipulating Robo expression in vivo perturbs commissural axon pathfinding in the chick spinal cord. *J Neurosci* 28 (35), 8698-8708 (2008).

Rela, L. & Szczupak, L., Gap Junctions: Their Importance for the Dynamics of Neural Circuits. *Mol. Neurobiol.* 30 (3), 341-358 (2005).

Ren-Ji Zhang, D.-J.Z.a.J.Z., Correlation of receptive field position of mechanosensory neurons and the strength of their connections to AP neurons in the CNS of the leech (Whitmania pigra) *Invertebrate Neuroscience* 1 (3), 249-254 (1995).

Reynolds, S.A., French, K.A., Baader, A., & Kristan, W.B., Jr., Staging of middle and late embryonic development in the medicinal leech, Hirudo medicinalis. *J. Comp. Neurol* 402 (2), 155-167 (1998).

Ristanovic, D., Milosevic, N.T., & Stulic, V., Application of modified Sholl analysis to neuronal dendritic arborization of the cat spinal cord. *J Neurosci Methods* 158 (2), 212-218 (2006).

Roerig, B. & Feller, M.B., Neurotransmitters and gap junctions in developing neural circuits. *Brain Res. Rev* 32 (1 Special Issue SI), 86-114 (2000).

Romano, N. & Macino, G., Quelling: transient inactivation of gene expression in Neurospora crassa by transformation with homologous sequences. *Molecular microbiology* 6 (22), 3343-3353 (1992).

Ruthazer, E.S., Akerman, C.J., & Cline, H.T., Control of axon branch dynamics by correlated activity in vivo. *Science* 301 (5629), 66-70 (2003).

Schwartz, T.H. *et al.*, Networks of coactive neurons in developing layer 1. *Neuron* 20 (3), 541-552 (1998).

Shen, K. & Bargmann, C.I., The immunoglobulin superfamily protein SYG-1 determines the location of specific synapses in C. elegans. *Cell* 112 (5), 619-630 (2003).

Sholl, D.A., Dendritic organization in the neurons of the visual and motor cortices of the cat. *J Anat* 87 (4), 387-406 (1953).

Siddall, M.E., Trontelj, P., Utevsky, S.Y., Nkamany, M., & Macdonald, K.S., Diverse molecular data demonstrate that commercially available medicinal leeches are not Hirudo medicinalis. *Proc Biol Sci* 274 (1617), 1481-1487 (2007).

Smith, C.L. & Frank, E., Peripheral specification of sensory neurons transplanted to novel locations along the neuraxis. *J Neurosci* 7 (5), 1537-1549 (1987).

Song, J. & Zipser, B., Kinetics of the inhibition of axonal defasciculation and arborization mediated by carbohydrate markers in the embryonic leech. *Dev Biol* 168 (2), 319-331 (1995).

Sperry, R.W., Chemoaffinity in the Orderly Growth of Nerve Fiber Patterns and Connections. *Proc Natl Acad Sci U S A* 50, 703-710 (1963).

Stuart, A.E., Physiological and morphological properties of motoneurones in the central nervous system of the leech. *Journal of Physiology* 209, 627-646 (1970).

Summerbell, D. & Stirling, R.V., The innervation of dorsoventrally reversed chick wings: evidence that motor axons do not actively seek out their appropriate targets. *J Embryol Exp Morphol* 61, 233-247 (1981).

Sun, P.Z., Schoening, Z.B., & Jasanoff, A., In vivo oxygen detection using exogenous hemoglobin as a contrast agent in magnetic resonance microscopy. *Magn Reson Med* 49 (4), 609-614 (2003).

Szabo, T.M., Faber, D.S., & Zoran, M.J., Transient electrical coupling delays the onset of chemical neurotransmission at developing synapses. *J. Neurosci* 24 (1), 112-120 (2004).

Tavazoie, S.F., Alvarez, V.A., Ridenour, D.A., Kwiatkowski, D.J., & Sabatini, B.L., Regulation of neuronal morphology and function by the tumor suppressors Tsc1 and Tsc2. *Nat Neurosci* 8 (12), 1727-1734 (2005).

Thompson, W., Kuffler, D.P., & Jansen, J.K., The effect of prolonged, reversible block of nerve impulses on the elimination of polyneuronal innervation of new-born rat skeletal muscle fibers. *Neuroscience* 4 (2), 271-281 (1979).

Thomson, E.E. & Kristan, W.B., Encoding and decoding touch location in the leech CNS. *J Neurosci* 26 (30), 8009-8016 (2006).

Torborg, C.L., Hansen, K.A., & Feller, M.B., High frequency, synchronized bursting drives eye-specific segregation of retinogeniculate projections. *Nat Neurosci* 8 (1), 72-78 (2005).

Tresch, M.C. & Kiehn, O., Motor coordination without action potentials in the mammalian spinal cord. *Nat. Neurosci* 3 (6), 593-599 (2000).

Tresch, M.C. & Kiehn, O., Synchronization of motor neurons during locomotion in the neonatal rat: Predictors and mechanisms. *J. Neurosci* 22 (22), 9997-10008 (2002).

Vrieseling, E. & Arber, S., Target-induced transcriptional control of dendritic patterning and connectivity in motor neurons by the ETS gene Pea3. *Cell* 127 (7), 1439-1452 (2006).

Wenner, P. & Frank, E., Peripheral target specification of synaptic connectivity of muscle spindle sensory neurons with spinal motoneurons. *J Neurosci* 15 (12), 8191-8198 (1995).

Wessel, R., Kristan, W.B., Jr., & Kleinfeld, D., Supralinear summation of synaptic inputs by an invertebrate neuron: dendritic gain is mediated by an "inward rectifier" K(+) current. *J Neurosci* 19 (14), 5875-5888 (1999).

Wiesel, T.N. & Hubel, D.H., Effects of Visual Deprivation on Morphology and Physiology of Cells in the Cats Lateral Geniculate Body. *J Neurophysiol* 26, 978-993 (1963).

Wiesel, T.N. & Hubel, D.H., Single-Cell Responses in Striate Cortex of Kittens Deprived of Vision in One Eye. *J Neurophysiol* 26, 1003-1017 (1963).

Wiesel, T.N. & Hubel, D.H., Comparison of the effects of unilateral and bilateral eye closure on cortical unit responses in kittens. *J Neurophysiol* 28 (6), 1029-1040 (1965).

Willard, A.L., Effects of serotonin on the generation of the motor program for swimming by the medicinal leech. *J Neurosci* 1 (9), 936-944 (1981).

Wong, W.T., Faulkner-Jones, B.E., Sanes, J.R., & Wong, R.O., Rapid dendritic remodeling in the developing retina: dependence on neurotransmission and reciprocal regulation by Rac and Rho. *J Neurosci* 20 (13), 5024-5036 (2000).

Yokoyama, N. *et al.*, Forward signaling mediated by ephrin-B3 prevents contralateral corticospinal axons from recrossing the spinal cord midline. *Neuron* 29 (1), 85-97 (2001).

Yuste, R., Peinado, A., & Katz, L.C., Neuronal domains in developing neocortex. *Science* 257 (5070), 665-669 (1992).

Zaidel, D.W., Quantitative morphology of human hippocampus early neuron development. *Anat Rec* 254 (1), 87-91 (1999).

Zlatic, M., Li, F., Strigini, M., Grueber, W., & Bate, M., Positional cues in the Drosophila nerve cord: semaphorins pattern the dorso-ventral axis. *PLoS Biol* 7 (6), e1000135 (2009).

Zoccolan, D. & Torre, V., Using optical flow to characterize sensory-motor interactions in a segment of the medicinal leech. *J. Neurosci.* 22, 2283-2298 (2002).