CELLULAR AND MOLECULAR CORRELATES

OF NEURAL MORPHALLAXIS IN *Lumbriculus variegatus*

A Dissertation

by

VERONICA GISELLE MARTINEZ

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2005

Major Subject: Zoology

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Approved as to style and content by:

Mark J. Zoran (Chair of Committee)	Vincent M. Cassone (Member)
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ABSTRACT

Cellular and Molecular Correlates of Neural Morphallaxis in *Lumbriculus variegatus*.

(May 2005)

Veronica Giselle Martinez, B.A.; B.A., University of St. Thomas

Chair of Advisory Committee: Dr. Mark J. Zoran

Tissue regeneration has intrigued biologists since the eighteenth century. While regeneration has been studied in many species, the cellular and molecular mechanisms governing successful compensation for lost body parts are poorly defined.

This dissertation examines the cellular and molecular correlates of a form of regeneration defined as morphallaxis. Morphallaxis does not involve cell proliferation, but instead relies on the reorganization of existing tissues to recover body structure and function. Morphallaxis is a mechanism used during segmental regeneration (i.e., head or tail replacement) by the aquatic oligochaete, *Lumbriculus variegatus*. Here, morphallaxis of the nervous system is documented during segmental regeneration of *Lumbriculus* and during asexual reproduction. The morphallactic processes, which underlie changes in the neural anatomy and physiology of these worms, are reminiscent of mechanisms utilized by other neural plasticity events, including learning and memory. Proteomic and biochemical studies focus on a molecular marker of neural morphallaxis. The expression patterns of morphallaxis-associated-protein 66, MP66, are differentially regulated during both regeneration and asexual reproduction. This expression pattern

correlates with time-points of major cellular changes associated with neural morphallaxis. Thus, cellular and molecular events, demonstrated as part of neural morphallaxis in *Lumbriculus*, are recruited in two life-history contexts. Chemical disruption experiments, where either segmental regeneration or asexual fission are blocked, reveal that morphallaxis can be mechanistically dissociated from regeneration and reproduction. These results set a foundation for future investigations of specific mechanisms that mediate this novel form of neural plasticity.

This work is dedicated to my grandmothers, Olivia Gomez and Josephina Arriaga, two of many women in my family whose strength and determination have imprinted every aspect of my life.

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I would like to especially recognize the support I have received from the Southern Regional Education Board (SREB) and the American Psychological Association (APA; Minority Fellowship Program Grant T32 MH-18882). Each of these organizations provided funding toward my stipend and tuition expenses as well as offering much needed advisement and support about the "realities" of academia. Additionally, image analysis was conducted in the Cellular Physiology and Molecular Imaging Laboratory at Texas A&M University, supported by NINDS (Grant PO1 NS-39546 to M.J.Z.).

These studies have taken me around the world, where I've met many wonderful 'oligochaetologists' or worm-people. Meeting them helped me feel less isolated and for their input I am grateful. I am especially appreciative of the input and guidance that I received throughout the project from Dr. Charles D. Drewes at Iowa State University. Charlie inspired me to remember my roots and go out to the field to really know what's

going on with my worm. I have been proud to carry on the elegant work that was started in his lab not so long ago. Many thanks to Dr. Jørgen Johansen, who generously donated all of the Lan 3-2 antibody needed to complete the work presented here. Jørgen also provided valuable input as I developed many of the molecular protocols used with *Lumbriculus*. I am also thankful for the many undergraduates that have assisted me over the past 6 years. You were helpful not only with the care and maintenance of animals but, with setting up of experiments. Many of you worked tirelessly, sometimes helping to prepare hundreds of worm fragments.

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the many laughs we shared. To the many friends I made in College Station, including Joshua Neunuebel, Sarah Bernhardt, and Brigitte Le Boeuf, thanks for being a wonderful support system. Additionally, I'd like to especially thank Dr. Deb Zoran for your support and understanding during the late night or weekend meetings Mark and I would have to schedule for revisions or experiments.

I am especially thankful for the love and support that I have received from my family not only through graduate school but throughout life. You instilled in me a passion for education which often resulted in tireless efforts to feed my need to know 'why.' More importantly you taught me that I cannot always assume that I know the answers to everything. For this and many other things you've taught me, I am very thankful. To my sister, Michelle, I am grateful for all the care you've given to our family especially when I could not be there to help. I love you more than words can express.

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CHAPTER I

INTRODUCTION

> Plasticity, then, in the wide sense of the word, means the possession of a structure weak enough to yield to an influence, but strong enough not to yield all at once.
> - William James, The Principles of Psychology.

The term 'plasticity' was introduced by William James to describe the innate ability of human behavior to adapt to changes in environment or state of being (James, 1950). Based on neural anatomical changes observed during transplantation and regenerative studies, Ramón y Cajal further proposed that this behavioral 'plasticity' might also have an anatomical basis (Ramón y Cajal, 1914). Since then, the phrase neural plasticity has been applied to short-term and long-term changes in the structure, function, and biochemistry of the nervous system. Plasticity is evident in such diverse phenomena as learning and memory, brain development, sprouting of axon terminals after a brain lesion, and various cellular forms of activity-dependent synaptic plasticity, such as long-term potentiation and long-term depression (Baudry, et al., 1999). Synaptic plasticity results in structural or functional change in a synapse, is often long-lasting. Examples of synaptic plasticity are not limited to classical studies of learning and memory, but are far-reaching, including the ability of song birds to learn specific song patterns (Woolley and Rubel, 2002); the spinal cord's ability to adjust its circuitry to varying loads, speeds, and directions during standing or stepping (Edgerton et al., 2004);

This dissertation follows the style and format of The Journal of Neuroscience.

and the olfactory system's ability to rapidly adapt to new odors (Wilson et al., 2004). Synaptic plasticity is also critical for the recovery of function following neural injury (Ambron and Walters, 1996; Matsukawa et al., 2004). Thus, studies of regeneration are also often models of synaptic plasticity.

Regeneration: A Form of Plasticity

Since the eighteenth century, the phenomenon of tissue regeneration has intrigued biologists (Dinsmore, 1991; Goss, 1991; Sanchez-Alvarado, 2000). Regeneration is widely distributed among most animal phyla although the degree of regenerative ability varies from species to species (Sanchez-Alvarado, 2000; Brockes et al., 2001; Stocum, 2004). Examples of regeneration are found in the urodele amphibians, noted as champions of vertebrate regeneration, which are able to regenerate a variety of structures including limbs, tail, ocular tissues, and heart tissue (Brockes et al., 2001; Chernoff et al., 2003). Even more remarkable regeneration occurs in invertebrate animals, such as some annelid worms, which can regenerate entire organisms from tissue fragments $1/50^{th}$ their original size (Morgulis, 1907; Berrill, 1952).

In 1901, T.H. Morgan recognized two modes of animal regeneration: morphallaxis and epimorphosis. Morphallaxis involves the transformation of existing body parts or tissues into newly organized structures without the need for new cell proliferation (Gilbert, 2000). An example of morphallactic regeneration is found in the classical regenerative model systems, *Hydra* and planaria. Classical studies with irradiated *Hydra* polyps demonstrated that head formation is not dependent on cell

division (Hicklin and Wolpert, 1973). Additionally, polyps treated with hydroxyurea (an S-phase blocking agent) prior to head amputation displayed no evidence of cell division within the regenerating heads (Cummings and Bode, 1984; Holstein et al., 2003). Thus, even without cell proliferation, a fully functioning head is still formed. Epimorphosis is a pattern of regeneration that involves the *de novo* generation of body parts or tissues. The cells for epimorphic regeneration can either be recruited by mobilization of a reserve population of stem cells (blastemal) or by de-differentiation of cells (non-blastemal) at the site of injury (Gilbert, 2000; Sanchez-Alvarado, 2000). Examples of epimorphic regeneration include limb regeneration in amphibians (Brockes et al., 2001) and compensatory regeneration of body organs (e.g., liver) in mammals (Stocum, 2004; Taub, 2004). Limb bud regeneration, first described by Spallanzani (Dinsmore, 1991), begins with the formation of a wound epidermis at the end of the regenerating stump where a blastema made up of dedifferentiated cells will form (Nye et al., 2003). The regeneration blastema resembles in many ways the progress zone of the developing limb. The dorsal-ventral and anterior-posterior axes between the stump and the regenerating tissue are conserved, and cellular and molecular studies have confirmed that the patterning mechanisms of developing and regenerating limbs are very similar. By transplanting regenerating limb blastemas onto developing limb buds, Muneoka and Bryant (1982) showed that the blastema cells could respond to limb bud signals and contribute to the developing limb. At the molecular level, just as Sonic hedgehog (Shh) is seen in the posterior region of the developing limb progress zone, it is seen in the early posterior regeneration blastema (Imokawa and Yoshizato 1997; Torok et al. 1999). The

initial pattern of Hox gene expression in regenerating limbs is not the same as that in developing limbs. However, the pattern of *Hoxa* and *Hoxd* gene expression during regeneration is characteristic of their expression patterns during limb development (Torok et al. 1998).

While observations of regeneration have been made in many species, the cellular and molecular mechanisms by which animals are able to successfully compensate for lost body parts are still poorly defined. The ultimate objective of regeneration research is to define those permissive and inhibitory factors that determine whether successful regeneration will occur. Thus, implications from regeneration research would greatly advance medical therapy and functional recovery following degenerative disease and injury. However, a caveat of this research is that the best model systems of regeneration, invertebrate and vertebrate alike, often have been ignored due to the lack of genetic tools for their manipulation. Additionally, genetic model systems currently display limited regenerative powers as seen in *Drosophila* imaginal disc regeneration (Milétich and Limbourg-Bouchon, 2000; Mattila et al., 2004) and murine central nervous system (CNS) regeneration (Brecknell and Fawcett, 1996; Caroni, 1998; Fenrich and Gordon, 2004). Despite these caveats, the ambition to induce tissue regeneration has been reawakened by a number of recent developments. The discovery of somatic stem cells suggests that adult tissues may have the latent capacity to regenerate (Stein, 2002; McKay, 2004). Additionally, cells from specific tissues can be grafted into many different adult organs, where they dedifferentiate and then recommit to form cells of different fates (Tosh and Slack, 2002). These advancements in

regeneration research, although inspiring, still do not match what has been learned with invertebrate models.

Regeneration occurs in a series of phases. The earliest phase includes the wounding event, wound healing, and blastema formation (Salo and Baguna, 2002; Holstein et al., 2003). These early events typically involve de-differentiation of the wounded tissue, the repatterning of existing cells, and/or the recruitment of naïve cells (stem cells) to form the blastema. BrdU labeling experiments in planaria have demonstrated that blastema formation following wound healing involves the continuous incorporation of a unique set of stem-cells, the neoblasts (Newmark and Sanchez-Alvarado, 2000; Reddien and Sanchez-Alvarado, 2004). Each successive step involved during regeneration requires the activation (or reactivation) of a parallel series of molecular events. For example, positional information in regenerating planarian fragments is determined immediately following injury and involves the up- and down-regulation of various Hox genes (Orii et al., 1999; Salo and Baguna, 2002). Perhaps most exciting has been the progression of invertebrate regeneration studies into a genetic era with the emerging technologies available for such research today. Techniques for studying gene function, such as RNAi (Sanchez-Alvarado and Newmark 1999; Newmark et al., 2003; Baker and Macagno, 2001) and in situ hybridization (Umesono et al. 1997), combined with the characterization of a large number of cDNAs from planaria (*Schmidtea mediterranea*; Sanchez Alvarado et al. 2002), hydrazoans (Hostein et al., 2003), and annelids (*Hirudo medicinalis*; Korneev et al., 1997; Baker and Macagno, 2001) have allowed the initiation of molecular genetic studies of regeneration.

Interestingly, many of the genes involved in regeneration have also been identified as homologues of genes expressed during embryonic development in both invertebrates and vertebrates. In *Hydra*, crucial genes of early embryogenesis are reactivated during regeneration (Holstein et al., 2003). Members of the Wnt signaling pathway, Wnt, β-Catenin, and Tcf, are all transcriptionally upregulated during early head bud formation and head regeneration in cnidarians (Hobmayer et al., 2000; Kusserow et al., 2005). Thus, key molecular players underlying regenerative mechanisms likely represent conserved developmental programs.

Neural Regeneration

The Mammalian CNS

An area of medicine that stands to benefit most from current regeneration research is that of brain and spinal cord injury. Ironically, this is an area of research where differences in regenerative potential among invertebrate and vertebrate animals are especially evident. Ramon y Cajal (1928) was the first to note that in the adult mammalian central nervous system,

> once the development was ended the fount of growth and regeneration of the axons and dendrites dried up irrevocably. In adult centers the nerve paths are something fixed, ended, immutable….nothing may be regenerated.

Indeed, following injury to the spinal cord, higher vertebrates are permanently paralyzed in body regions that are controlled by the damaged area (Puchala and Windle, 1977; Goldberg and Barres, 2000; David and Lacroix, 2003). It is now known that lesions within the adult CNS are able to trigger some degree of regeneration (David and

Aguayo, 1981) and that the success of functional recovery is decreased by the presence of an inhibitory environment within the CNS.

The success of regeneration is determined largely by the presence or absence of inflammation or scar tissue between the proximal and distal segments of the injured nerve (Brecknell and Fawcett, 1996; Stocum, 2004). Following various lesions of the spinal cord, damaged axons produce growth cones, specialized, amoeba-like structures at the tip of an axon, filled with microtubules that help in process elongation. However, these newly sprouted growth cones make poor progress through inflammatory tissue and are immobilized by bacterial infections in the repairing wound (Brecknell and Fawcett, 1996). Moreover, it has been demonstrated that some CNS neurons, for example retinal ganglion cells (RGCs), possess the ability to regenerate early-on in development, but lose this ability following a critical period usually correlated to the onset of myelination (Saunders et al., 1998; Goldberg et al., 2002). Axonal growth inhibiting molecules are also released by the CNS myelin (Nogo-A, Myelin-Associated Glycoprotein, and Oligodendrocyte Myelin Glycoprotein) following injury (He et al., 2003; He and Koprivika, 2004; Chen et al., 2000; Filbin, 2003; Wang et al., 2002). While it is generally accepted that these inhibitory properties of the CNS contribute importantly to the lack of successful neural regeneration, it is also important to recognize that these injured CNS neurons may not be receiving injury-induced trophic stimuli needed to promote the survival of neurons (Goldberg and Barres, 2000). These trophic stimuli may include molecules that play important roles during neurogenesis in early development. Thus, the failure of the CNS to regenerate following injury is a multi-

faceted issue whose complexity has made it difficult to identify specific mechanisms associated with successful neural regeneration.

The Invertebrate CNS

In contrast to the mammalian CNS, the invertebrate CNS is far less complex and possesses tremendous capacities for neural regeneration. While regeneration within the vertebrate CNS occurs, especially in fishes (Mackler and Selzer, 1985; Matsukawa et al., 2004), amphibians (Lee, 1982; Clarke et al., 1988; Chernoff et al., 2003), and some avian species (Morest et al., 2004; Roberson et al., 2004), many more examples of nervous system regeneration exist for invertebrate systems. Of these systems, studies carried out in gastropod molluscs (e.g., *Helisoma* and *Aplysia*) have been most revealing of the mechanisms governing neural regeneration and synaptic plasticity (Moffet, 1995; Kandel, 2001). The gastropod nervous system responds to body injury by releasing trophic factors that may promote repair as well as bring about changes within the nervous system (Moffett, 1995; Dulin et al., 1995; Spira et al., 2001). In *Aplysia*, signals generated by axon injury trigger and prime transcription-dependent responses, such as growth and long-lasting compensatory changes in neuronal excitability (Ambron and Walters, 1996). It is also thought that the repair of an injured axon is initiated, maintained, and completed in a series of phases; the earliest phase composed primarily of the initial signals received by the nucleus following injury (Ambron et al., 1992; Dulin et al., 1995; Gunstream et al., 1995). In general, the earliest responses to neuronal injury include the depolarization and electrical discharges that reflect membrane disruption. This membrane depolarization sends rapid signals to the soma and causes

release of neuromodulators (Glanzman et al., 1989a; b). Additionally, ion entry at the site of injury, specifically Ca^{2+}, can lead to many changes, including activation of enzymes that generate retrograde signals or alter the cytoskeleton so that membrane sealing can occur (Yawo and Kuno, 1983; 1985; Meiri and Grafstein, 1984; Walters and Ambron, 1995; Moffet, 1995). Interestingly in *Aplysia*, serotonin is elevated in response to injury and leads to cAMP-dependent signaling (Azhderian et al., 1994; Walters and Ambron, 1995). Serotonin also modulates neurite outgrowth during CNS development (Haydon et al., 1984; Goldberg and Kater, 1989; Diefenbach et al., 1995) and regeneration (Murrain et al., 1990) of *Helisoma* neurons. Moreover, this modulation of growth cone motility by serotonin is altered by a sustained depolarization and Ca^{2+} influx (Cohan et al., 1987; McCobb and Kater, 1988; Price and Goldberg, 1993). Interestingly, these developmental events are reminiscent of the processes that underlie simple forms of learning and memory in molluscs (Moffet, 1995; Walters, 1994; Sung and Ambron, 2004; Sung et al., 2004; Weragoda, et al., 2004). For example, changes in sensory neuron spike threshold, spike shape, and accommodation rate following injury are similar to changes observed during classical conditioning (Walters, 1994). Thus, learning and memory, regeneration, and development share cellular and molecular mechanisms for the generation of this triad of neural plasticity.

Although many tissues have been implicated as sources of signals that trigger regeneration (Bauduin et al., 2000), studies in flatworms (planaria) and annelid worms have suggested a specific role for neural tissue in the activation of general regenerative mechanisms (Kishida and Kurabuchi, 1978; Reuter and Gustafsson, 1996; von Bernhardi

and Muller, 1995; Baker and Macagno, 2001; Bueno et al., 2002; Müller et al., 2003; Müller, 2004b). Initiation of segmental regeneration by the nervous system has been hypothesized for annelid worms (Müller et al., 2003). In support of this idea, removal of portions of the nerve cord in *Eisenia foetida* (Herlant-Meewis, 1964) and *Eurythoe complanata* (Müller et al., 2003; Müller, 2004b) delayed the ensuing epimorphic regeneration of a new head or tail. Additionally, serotonin, produced by neurons of the CNS, is thought to play a role in activation of cell proliferation in the wound blastema (Müller et al., 2003). Specifically, the appearance of serotonergic fibers in the regenerating blastema, was paralleled by a similar arrangement of the dividing cells (Müller et al., 2003). In Chapter IV, ectopic head studies provide another example of the importance of the nervous system during head regeneration. Thus, combined experimental results across various invertebrate phyla suggests that the nervous system is a site of important regulators of regeneration, either morphogenic or physiogenic.

A Model System for Neural Regeneration

Lumbriculus variegatus possess a remarkable capacity for regeneration. Worm fragments generated either as a result of injury or asexual fragmentation replace missing heads or tails via epimorphic regeneration and coordinate this process with morphallactic regeneration within original body segments. In these dissertation studies, I have characterized the mechanisms (cellular, molecular, and physiological) that regulate morphallaxis within the CNS of *Lumbriculus*.

A Life History of Lumbriculus variegatus

Lumbriculus is a freshwater oligochaete of the Order Lumbriculida (Brinkhurst and Jamieson, 1971; Jamieson, 1981; Brinkhurst and Gelder, 1991) which is commonly called the California blackworm or mudworm. Composed primarily of mud-dwelling animals, lumbriculids are distinct from other freshwater oligochaetes, such as tubifex worms (Order Tubificida) and terrestrial oligochaetes, the earthworms (Order Haplotaxida) and are thought to have evolved as an early branch of the annelid phylogenetic tree (Brinkhurst and Jamieson, 1971; Jamieson, 1981).

Lumbriculus is found throughout North America and Europe in shallow ponds, lakes, and marshes where it feeds on decaying vegetation and microorganisms (Brinkhurst and Jamieson, 1971; Jamieson, 1981). Worms at various stages of development can easily be collected during the spring and early summer months; commonly found beneath layers of decomposing leaves or sediments near the base of emerging vegetation, such as cattails. *Lumbriculus* may also be found in silt sediments of deeper water, but these niches are not as prevalent as littoral zones. Typically, a worm can be found with its head inserted into a burrow and its tail extended up into the water column with the tip of the tail just breaking the air/water interface. *Lumbriculus*' most caudal tail segments are specialized for gas exchange. The dorsal body wall of these tail segments is notably devoid of circular and longitudinal muscles, providing special access of the dorsal blood vessel to gas exchange across the epidermal layers (Drewes, 1990). Tails are bent to run parallel with the water surface such that the very tip of the tail is at a right angle with the rest of the worm's body. Thus, this characteristic

tail positioning results in the placement of the enlarged dorsal blood vessel in close proximity to the air (Drewes, 1990). This behavior is different from that observed in tubificid worms (*Branchiura sowerbyi*) which undulate gill filaments on their tail ends as they protrude from their burrows (Stephenson, 1912; Kawaguti, 1936; Drewes and Zoran, 1989).

Lumbriculus ranges in size from about 5 to 10 cm (100-250 segments) in length, depending upon their developmental state. Sexually mature worms are typically larger and can be up to 1.5 mm in diameter (Drewes and Brinkhurst, 1990). Worms raised in the laboratory are usually smaller in size (100-150 segments or 4-6 cm in length) and do not reach sexual maturity. Lumbriculid worms are hermaphroditic, containing both male and female reproductive organs within segments 10–17 (Jamieson, 1981). As clitellate annelids, lumbriculid worms utilize contact mating and directly exchange sperm during a pseudocopulation event (Shankland and Savage, 1997). Fertilization then takes place at a later time outside of the worm (Brusca and Brusca, 1990; Shankland and Savage, 1997). Transparent cocoons, each containing 4-11 fertilized eggs, then undergo direct embryonic development with no larval stage (Anderson, 1973; Drewes and Brinkhurst, 1990). Small worms (about 1 cm in length) emerge from cocoons in about two weeks.

Worms cultured in the laboratory reproduce via asexual reproduction, as they do throughout the summer and fall months in nature. Asexual reproduction in *Lumbriculus* is described as architomic fission (Stephenson, 1930; Berrill, 1952; Brusca and Brusca, 1990). Architomy is defined by the production of body fragments, in this case worm fragments, whose fragmented ends must then develop a new head and or tail via

epimorphic regeneration (Morgulis, 1907; Drewes and Fourtner, 1991). Thus, the end result of asexual fragmentation is the production of two or more zooids (clones) of the original adult. The observations described in this dissertation regarding asexual reproduction in *Lumbriculus* defines many aspects of this process that are not well understood (Chapter II).

Lumbriculus is also able to self-amputate in response to the threat of injury or other noxious stimulation via a process called autotomy (Lesiuk and Drewes, 1999). Sudden compressive stimuli of the sensory epithelium result in autotomy or rapid self fragmentation. This ability to autotomize provided challenges during the design of experiments discussed here, which at times called for invasive manipulation of the animal. Thus, pharmacological agents (such as nicotine) were utilized as a paralytic to reduce autotomy reflexes (Lesiuk and Drewes, 1999). It is important to note that autotomy, although involving fragmentation, is fundamentally distinct from the regulated reproductive process of architomy.

Lumbriculus exhibits anterior-posterior gradients in anatomy and physiology. In an adult worm, anterior segments are darkly pigmented and wider than posterior segments. Additionally, head segments are distinguished from the rest of the worm's body by the presence of a prostomium (mouth) in the first segment and a pharynx as well as reproductive organs (in sexually mature worms) within the first 17 segments (Jaimeson, 1981). Although posterior segments appear similar, there are some noted differences. For example, the posterior most segment is unique in that it contains the anal opening and the terminal 20 segments are thought to include, along with respiratory

adaptations, a large population of photoreceptor cells which are used in detecting potential predatory threats (Drewes and Fourtner, 1989; Drewes, 1990; Jaimeson, 1981). Moreover, unlike leeches and earthworms, segments in *Lumbriculus* are not terminally differentiated in mature worms. Thus, body segments at any position along the length of the anterior-posterior axis retain the ability to change their positional identity as is described during regenerative or reproductive processes (Drewes and Fourtner, 1990; Lesiuk and Drewes, 2001a, Chapter II).

Lumbriculus also exhibits anterior-posterior gradients in behavior that are easily monitored (Drewes and Fourtner, 1990; Lesiuk and Drewes, 2001a). With its tail extended into the water column, *Lumbriculus* is exposed to predation and thus has evolved rapid escape reflex behaviors that aide in survival tactics (Drewes, 1984; Zoran and Drewes, 1987). Specifically, stimulation of segments in the posterior 2/3 region of the worm's body results in posterior shortening or tail withdrawl (Drewes, 1984; Zoran and Drewes, 1987; Drewes and Fourtner, 1989; Drewes and Fourtner, 1990). Also, touch-stimuli applied to segments found in the anterior 1/3 region of the worm's body result in a quick anterior shortening or head withdrawl (Drewes, 1984; Zoran and Drewes, 1987; Drewes and Fourtner, 1990). Stimulation of anterior segments also results in a 180° turn or reversal locomotor response away from the aversive stimulus, where as stimulation of posterior segments elicits rapid undulating swim movements (Drewes, 1999). These behaviors, which are specifically activated by anterior- or posterior-specific sensory inputs, are also mediated by motor networks specific to body regions.

Oligochaete Nervous System Anatomy and Physiology

The oligochaete central nervous system generally consists of a cerebral ganglion (brain; a fused supra-esophageal ganglion) which is located in segment 1, and is connected to the sub-esophageal ganglion and subsequently a ventral nerve cord (VNC) via two circumesophageal connectives (Stephenson, 1930; Bullock, 1965; Jamieson, 1981). In lumbriculid worms, the VNC extends down the length of the worm and gives rise to four pairs of segmental nerves within each segment (except segments 1 and 2; Bullock, 1965). These segmental nerves extend laterally around the body wall and are the source of synaptic input (sensory) and output (motor) within the oligochaete's CNS (Stephenson, 1930; Bullock, 1965; Jamieson, 1981). Groups of different types of neurons (sensory, motor, and interneurons) converge and are organized within each segment of the VNC (Jamieson, 1981). Axons of some of these sensory and motor neurons extend through the segmental nerves, while others extend into the neuropile of the VNC. Thus, the neuropile is a site of integration of many synaptic events that underlie the function of the worm's neuronal circuits controlling behavioral reflexes (Bullock, 1965; Jamieson, 1981).

A conserved feature of virtually all oligochaetes is the presence of three giant fibers (Fig. 1), located in dorsal regions of the ventral nerve cord (Bullock, 1965; Jamieson, 1981; Zoran and Drewes, 1987). There is a marked diversity in the number, size and arrangement of the giant fibers within the annelids (Bullock, 1965). In polychaetes, this variation is prevalent throughout the entire class with some representatives having no giant fibers (Family Syllidae) and others having highly

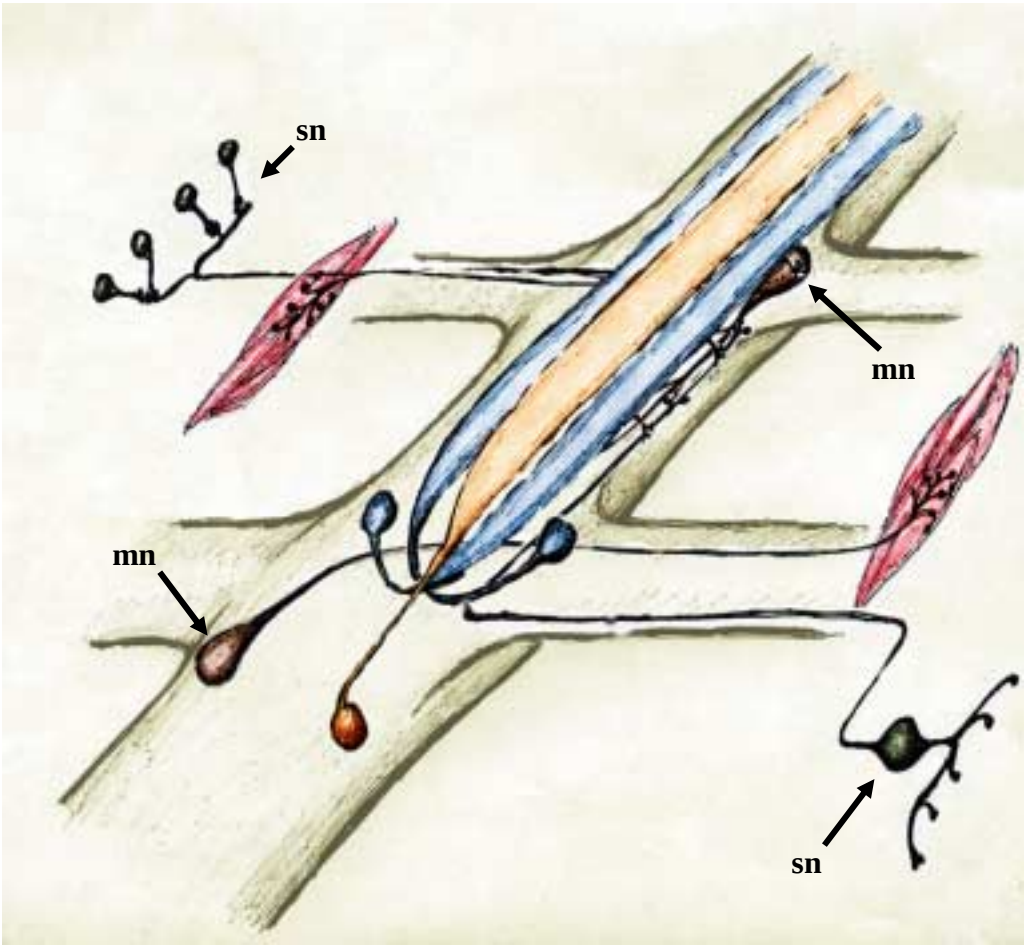

Figure 1 – Oligochaete CNS anatomy.

The oligochaete CNS is composed of a ventral nerve cord (VNC). The dorsal region of the VNC of oligochaete worms contains three giant nerve fibers: one medial and a pair of lateral giant fibers. Each MGF axon (yellow) has ventrally projecting collaterals and one cell body. Collaterals also project ventrally from each LGF axon (blue). One of these collaterals, after crossing the VNC midline becomes the neurite of the LGF cell body. In each segment (except segments 1 and 2) the VNC gives rise to pairs of segmental nerves. Some fibers within these nerves are sensory in function (sensory neuron; sn) and other fibers serve as motor neurons (mn) and innervate the musculature within the body wall. Drawings by MJ Zoran.

elaborated structures, including giant fibers derived from numerous cell bodies in anterior segments and other more simple configurations including a single paired giant fiber derived from two cell bodies (Nicol, 1948; Bullock, 1965). In the oligochaetes, there is less variation in giant fiber number and anatomy with few exceptions (e.g.,

Limnodrilus hoffmeisteri; *Branchiura sowerbyi*; Zoran and Drewes, 1987). Each of these giant nerve fibers is derived from a chain of giant axons which arise from segmentally arranged interneurons whose cell bodies are found just ventrally within the neuropile (Bullock, 1965; Günther and Walter, 1971; Jamieson, 1981). These three giant fibers include one medial (MGF) and a pair of lateral giant (LGF) axons (Fig. 2). Giant axon dye-filling in *Lumbricus* demonstrates that these axons are septate in nature;

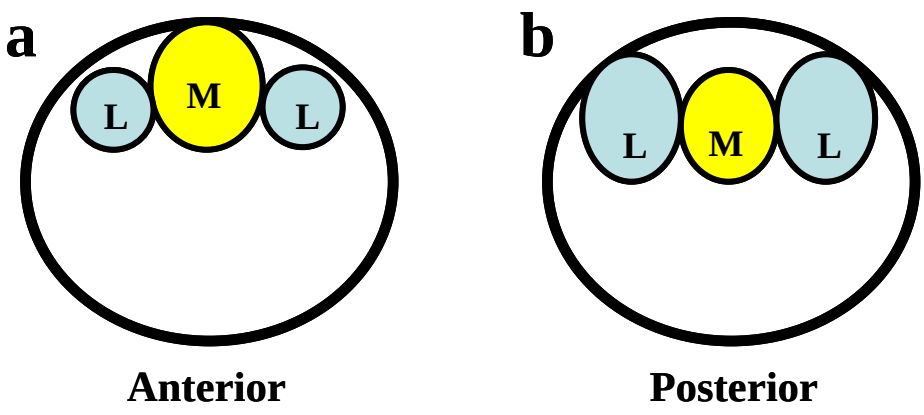

Figure 2 – Giant fiber diameter in anterior and posterior body regions.

a. and b. Cross sections of the VNC. a. In the anterior body region, the MGF (M) is much larger in diameter than the two LGFs (L). b. In the posterior body region the opposite is true and the two LGFs are larger in diameter than the MGF.

having distinct, segmental divisions separated by a membranous septum (Appendix A), as opposed to being syncytial, where there are no cellular divisions and thus a continuous cytoplasm between cells. Moreover, each segmentally arranged giant axon is connected via gap junctions (i.e., electrically coupled) allowing for uninterrupted through-conduction of nerve impulses along the length of the giant fiber system (Mulloney, 1970; Brink and Ramanan, 1985). Each giant fiber (GF) has 2-4 ventrally

projecting collaterals and one cell body per segment (Fig. 1). Additionally, in most oligochaetes, one lateral giant fiber (LGF) collateral forms a cross-bridge with the contralateral LGF within each segment. These interconnections are undoubtedly the basis for observed electrotonic coupling between the LGFs and the resultant bilateral synchronization of LGF action potentials during spike propagation (Drewes, 1984). It has also been demonstrated that lumbriculid giant fiber axons are ensheathed by glial cell membranes, resulting in myelin-like layers surrounding the axons (Günther, 1976; Roots and Lane, 1983; Appendix A). The presence of myelin-like insulation on giant fiber axons is thought to be the basis of observed rapid escape reflexes and thus functions to increase conduction velocity along the length of the giant fibers (Zoran et al., 1988; Drewes and Fourtner, 1990).

Giant Fiber Pathways Mediate Rapid Escape Reflexes

Rapid escape reflexes initiated following noxious stimulus (i.e., a potential predatory threat) are mediated by the giant fiber pathways. Activation of these giant fibers via sensory stimuli (e.g. tactile or photic) results in the rapid conduction of nerve impulses down the length of the fiber that, in turn, activate motor neurons, which impinge upon longitudinal muscles responsible for body shortening (Drewes, 1984; Drewes and Fourtner, 1989; Drewes and Brinkhurst, 1990). Moreover, these rapid escape reflexes are differentially regulated by the medial and lateral giant fibers. That is, head withdrawal reflexes, in response to sensory stimuli to the anterior 1/3 of the body, are governed by the medial giant fiber (MGF) and tail reflex responses are governed by the lateral giant fibers (LGF; Drewes and Fourtner, 1990; Lesiuk and Drewes, 2001a).

Interestingly, there are a few segments (segments 38-58 in a worm of 150 segments) in which both a head and tail withdrawl can be elicited and both MGF and LGF activation is detected (Drewes and Fourtner, 1990). Thus, giant fiber function is governed by discrete sensory fields, with the anterior 1/3 body region falling within the MGF sensory field (MGF s.f.) and the posterior 2/3 body region comprising the LGF sensory field (LGF s.f.). Interestingly, although these three giant fibers are conserved among virtually all oligochaetes, there is a fundamental difference in these rapid escape pathways between terrestrial worms (most susceptible to anterior predatory attack) and aquatic worms with tails extended from the substrate burrows (susceptible to posterior attack). Specifically, LGF sensory fields, giant fiber diameters, conduction velocities, and synaptic efficacies have become highly adapted for speed during aquatic worm (tubificid and lumbriculid) evolution (Zoran and Drewes, 1987).

Regeneration in Lumbriculus

Lumbriculus, like many invertebrates, possesses an incredible capacity for regeneration. However, *Lumbriculus* is among an elite few which seem to have virtually unlimited capability for regeneration. For example, although many annelid worms are limited in their ability to regenerate anteriorly, this regenerative capacity is present in some species of worm like *Lumbriculus* (Berrill, 1952). Regeneration in annelid worms takes place to different extents and usually varies according to the position of the cut along the anterior-posterior axis (Berrill, 1952). For example, in *Eisenia foetida* (Lumbricidae) regeneration of anterior structures is drastically reduced as more segments are removed. Consequently, *Eisenia* is not capable of regenerating

head segments when greater than 20 segments are removed (Moment, 1950; Berrill, 1952). However, in *Lumbriculus*, 7-8 segments are regenerated from any segmental position along the length of the animal (Berrill, 1952; Drewes and Fourtner, 1990; Chapter II). Regeneration of posterior segments, although more common, is also highly variable among the oligochaetes (Berrill, 1952). Successful regeneration of posterior segments relies on the migration of stem cells (neoblasts).

The process of head regeneration involves three phases: wound healing, blastema formation, and growth and differentiation (epimorphosis) of the blastema (Morgulis, 1907; Berrill, 1952). Wound closure is primarily the result of the contraction of the body wall musculature directly after amputation (Christensen, 1964; Lesiuk and Drewes, 1999). The wound is subsequently covered by a transparent blastema which differentiates (epimorphosis) via proliferation of stem cell populations (neoblasts), or dedifferentiation of existing cells, into the missing body parts (Berrill, 1952; Stone, 1932; Michel, 1898).

Perhaps even more remarkable among annelid regeneration is the ability to regenerate both anterior and posterior body parts from tiny body fragments. While much of the current literature documents this ability to regenerate from body fragments in polychaete worms, much less is known about its occurrence in the oligochaetes (Berrill, 1952). Nonetheless, *Lumbriculus* displays an extensive ability to regenerate a complete adult animal following reduction to as little as three segments (Morgulis, 1907; Berrill, 1952). Regeneration from body fragments (segmental regeneration) in *Lumbriculus*, involves both epimorphosis and morphallaxis. Segmental regeneration of new head and

tail segments involve the processes of anterior and posterior epimorphic regeneration as described above. Worm fragments regenerate 7-8 head segments and varying lengths of tail (Berrill, 1952; Drewes and Fourtner, 1990; Chapter II). This characteristic regeneration of a short 7-8 segment head results in positional transformations for fragments removed from the posterior-most segments such that following the regenerative process, these posterior fragments are now more anteriorly located (Drewes and Fourtner, 1990; Chapter II). The work presented in this study exploited this switch in positional identity during segmental regeneration, to study the cellular and molecular correlates of morphallactic regeneration in *Lumbriculus* (Fig. 3).

Morphallactic Regeneration

Morphallactic regeneration has been demonstrated in various species of invertebrates, the classic model being *Hydra* (Holstein et al., 2003). Another well-studied example of morphallaxis is found in pharyngeal regeneration in planarian flatworms (Reddien and Sanchez-Alvarado, 2004). Following isolation of planarian tails, a new head is produced and within the original tail fragment a new pharynx is produced (Morgan, 1901). The new pharynx arises in an area posterior to that of the original pharynx and previously contained other structures of the gastrovascular system (Reddien and Sanchez-Alvarado, 2004). Thus, the isolated tail fragment reorganizes the identity of some of its tissues in order to function appropriately following regeneration of a new head (Morgan, 1901). Other examples of morphallaxis are found in the sea cucumber, *Holothuria forskali* (VandenSpiegel et al., 2000) and in various species of oligochaetes.

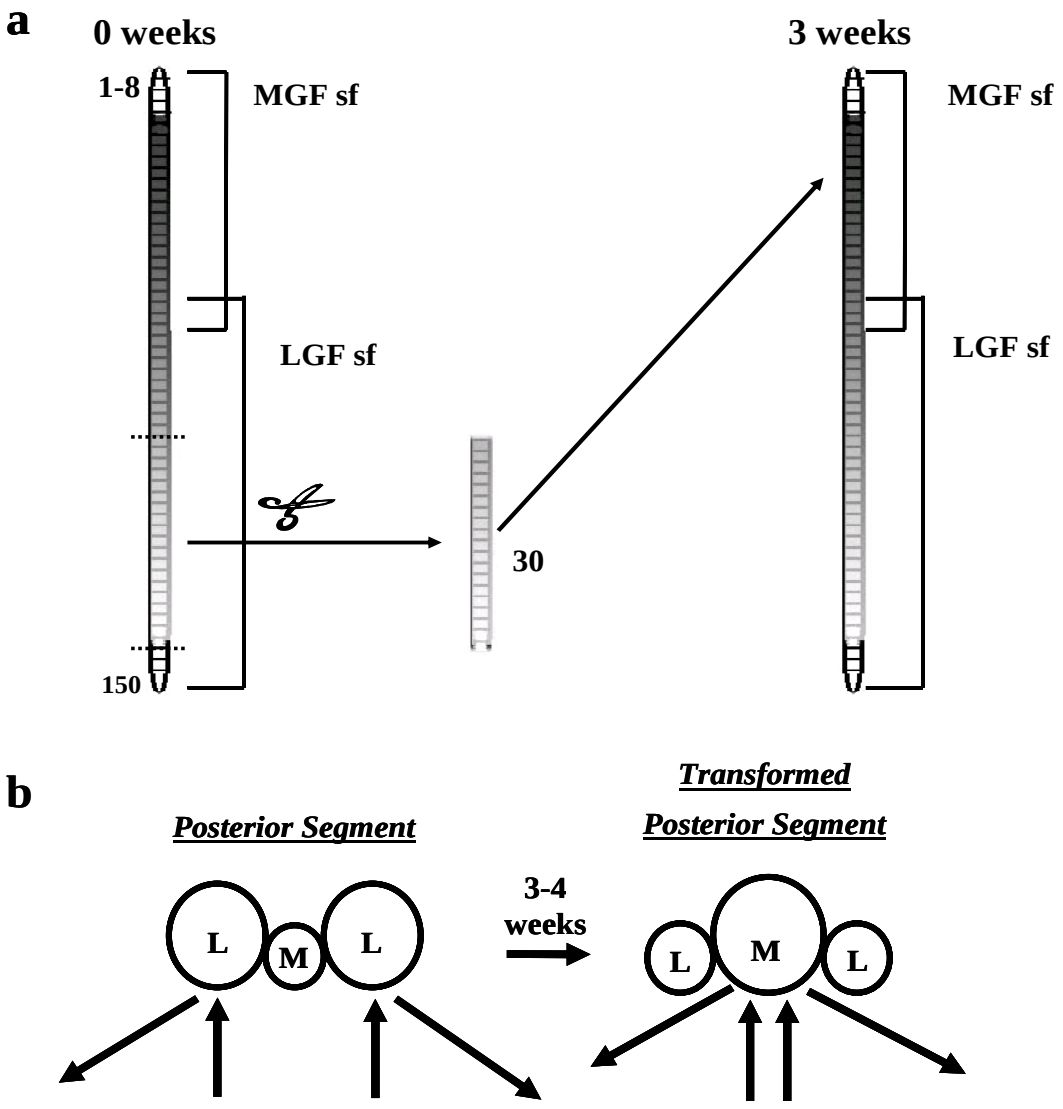

Figure 3 – Neural morphallaxis in L.variegatus*.*

a. Removal of a 30 segment fragment, from the posterior region of a worm, results in structural and physiological reorganizations within the central nervous system, which are appropriate for the fragment's new anterior positional identity. (See Chapter II)

b. Morphallaxis in Lumbriculus *describes anatomical (giant fiber diameter) and physiological (sensory inputs/outputs) changes that occur within the giant fiber system of the worm. This reorganization is especially evident in posterior regenerating fragments. M=MGF; L=LGF. Arrows indicate sensory input and motor output.*

Studies of regeneration in earthworms have focused largely on CNS and axonal regeneration, characterized by neuronal processes arising within damaged nerves (Birse and Bittner, 1981; Drewes et al., 1988; Lyckman et al., 1992). However, segmental regeneration (epimorphosis) in these lumbricid worms (earthworms) is rare. Evidence of morphallactic regeneration in oligochaetes has only been documented in only three species, *Lumbriculus variegatus* (Drewes and Fourtner, 1990; Chapter II), *Eisenia foetida* (Chapron, 1970), and *Enchytraeus japonensis* (Yoshida-Noro et al., 2000; Myohara et al., 1999; Myohara, 2004; Müller, 2004b). Drewes and Fourtner (1990) described morphallaxis of the medial giant fiber and lateral giant fiber systems in *Lumbriculus* during segmental regeneration of worm fragments. Following isolation of both anterior and posterior fragments, sensory fields within these segments are reorganized based on their new position within the regenerating worm. MGF and LGF sensory fields are functionally and anatomically reorganized segments of the original fragment. Therefore, changes in GF sensory fields have been utilized in this dissertation to demonstrate GF reorganization during morphallaxis. Specifically, fragments, originally subserved by a particular sensory field, gradually become subserved by the other, as new head and tail segments are regenerated (Drewes and Fourtner, 1990). This behavioral morphallaxis is also accompanied by appropriate structural and functional changes within the giant fiber pathways. Changes in giant axon diameter and conduction velocity accompany changes in rapid escape reflex behaviors (Drewes and Fourtner, 1990) and reflect the fragment's acquisition of a new positional identity following morphallaxis. Again, I have used these characteristic structural, physiological,

and behavioral changes to study the associated cellular and molecular changes that occur during neural morphallaxis.

Neural Regeneration: Cellular and Molecular Mechanisms

In studies of both vertebrate and invertebrate animal systems, a basic goal is to understand how regeneration is initiated, what sets of genes are involved, what morphogens, growth factors and hormones might play a role, and what cell-to-cell interactions occur in order to regulate the highly specific replacement of missing body parts in such a way that they function appropriately. These are important, wide-ranging and complex issues of developmental biology. On a more narrow scale, regeneration of neural networks provides most of these same challenges. Re-establishment of specific synaptic connections following damage involves the orchestration of various cellular and molecular cues. Since the fundamental works in neural development of Ramon y Cajal (1890) and Roger Sperry (1963), many molecules have been demonstrated as components of process outgrowth and synaptic plasticity. Moreover, the same cellular events and molecular cues involved during development also seem to play similar roles during neural regeneration (von Bernhardi and Muller, 1995; David and Lacroix, 2003; Huber et al., 2003; Chernoff et al., 2003).

Cellular Correlates of Neural Regeneration

In many animals, damage to the nervous system is compensated for with new neurogenesis (e.g. ablation of ganglia). However, if only an axonal process is damaged this activates a series of events which may not involve neurogenesis but instead involve

wound healing followed by emergence of a new growth cone. Ramon y Cajal was the first to suggest that growth cones must play a role in navigation toward synaptic targets. Growth cone movement is accomplished through the use of finger-like extensions of filopodia (microspikes), which are connected by a thin, outer covering of membrane, called the lamellipodia. Growth of the axon occurs when a filopodium extends and then remains in place while the lamellipodium advances toward it, giving growth cones their characteristic "amoeboid-like" appearance. This movement is mounted by the continual cycle of polymerization and depolymerization of actin filaments at the leading edge of the growth cone in addition to microtubule assembly and disassembly (Forscher and Smith, 1988; Suter and Forscher, 2000; Gallo and Letourneau, 2000; Spira et al., 2001).

Finally, the regenerating axon will complete its extension as it nears its specified target. The selection of potential synaptic targets and the formation of synaptic connections require on-going communication between pre- and postsynaptic partners, as well as a number of signaling systems which are involved at various stages of these processes (Tessier-Lavigne and Goodman, 1996; Sanes and Scheller, 1997; Davis and Goodman, 1998; Holt and Harris, 1998). Studies of the embryonic neuromuscular junction have provided evidence of a list of both anterograde signals from the presynaptic neuron that affect differentiation of the muscle target (Sanes and Scheller, 1997) and retrograde signals from the postsynaptic target that affect differentiation of the nerve terminal (Fitzsimonds and Poo, 1998). Thus, a variety of molecules within the target zone, including gradients of ephrins and CAMs, combinatorialy encode different possible target cells along various axes and layers (Scheiffele, 2003).

Having completed target selection does not always lead to the formation and/or stabilization of a synapse. It is now known that many postsynaptic targets are initially innervated by multiple axons but, only one will 'win' and be further strengthened. Stabilization of synapses not only requires the encouragement of survival by various trophic factors (e.g., NGF, BDNF), but also requires the appropriate pre- and postsynaptic modifications (i.e., synaptic machinery) to be present and functional (Hamburger and Levi-Montalcini, 1949; Sanes and Scheller, 1997). This later phase of synapse formation can thus be considered one of synaptic refinement which involves signaling events and activity-dependent processes that refine the synaptic circuit.

Molecular Correlates of Neural Regeneration

These cellular processes of neural regeneration, as in early development, must be highly orchestrated. Recent biochemical and genetic studies have identified evolutionarily conserved ligand-receptor systems involved in neural development and synaptic plasticity (Tessier-Lavigne and Goodman, 1996; Goodman and Shatz, 1993). They include adhesion molecules, guidance molecules, and neurotrophins which provide directional information during neural development (Tessier-Lavigne and Goodman, 1996; Song and Poo, 1999; Huber et al., 2003; Table 1). Interestingly it is now understood that these molecules are highly conserved across species, including vertebrates and invertebrates (Goodman, 1994) and are reactivated during neural regeneration.

Regeneration thus must involve a cascade of molecular signaling events that are similar to what occurs during early development. There are early signals which include

expression of axial patterning genes, like Hox genes (Orii et al., 1999; Thorndyke et al., 2001; Salo and Baguna, 2002) and there are genes which are expressed during neurite outgrowth which provide essential guidance mechanisms for the regenerating axon. Studying regenerating axons from retinal ganglion cells (RGCs) in amphibians, Roger Sperry (1963) noted that differential chemical attraction might be the explanation behind the specificity of growth cones for their targets. In his chemoaffinity hypothesis, Sperry (1963) was the first to suggest that growth cones utilized specific surface markers for pathway and target recognition. These guidance cues, either attractive or repulsive, include contact-mediated or secreted molecules which act over short or long distances, respectively (Tessier-Lavigne and Goodman, 1996; Huber et al., 2003; Table 1). Thus, by integrating a set of cues, growth cones are able to appropriately select the correct path for their target.

Lan 3-2, Carbohydrate Signaling and CAMs

Studies using the nervous system of embryonic and adult leeches have played an important role in our knowledge about growth cone dynamics and target recognition (Baker and Macagno, 2001). In 1981, a set of monoclonal antibodies generated against the leech CNS were developed (Zipser and McKay, 1981). It was later discovered that these monoclonal antibodies labeled specific neurons in the leech which previously could only be identified by morphology, position, and function (Zipser et al., 1994). Of the many antigens discovered using these antibodies, the most interesting appeared to be a 130 kDa molecule recognized by the antibody Lan 3-2. Lan 3-2 labeled the surface of neurons which were growing in bundles or fascicles in the central axon tract within the

Table 1- Molecular Factors That Shape Axon Guidance

Ligand	Receptors	Functions	References
Adhesion Factors			
fibronectin laminin	*Integrins*	*Growth cone guidance, axon fasciculation, target recognition*	*Hynes et al., 1987*
Cell Adhesion Molecules: L1 ApCAM LeechCAM	*CAMs, Integrins, RPTPs*		*Brummendorf & Rathjen, 1997; Crossin & Krushel, 2000*
Cadherins	*Cadherins*		*Sivasankar et al., 1999*
Guidance Factors			
Semaphorins	*Neuropilins, plexins,L1*	*Chemoattractant; Chemorepellant*	*Raper, 2000*
Netrins	*UNC-40 (c.elegans), DCC (verts.), Frazzled(D.mel), UNC-5 (repulsive)*	*Chemoattractant; Chemorepellant (UNC-5)*	*Chisholm & Tessier-Lavigne, 1999*
Ephrins	*EphA & Eph B*	*Regulation of Adhesion*	*Cooke & Moens, 2002; Holmberg & Frisen, 2002; Bagri et al., 2002*
Slit	*Robo*	*Chemorepellant*	
contactin, pleiotrophin? midkine?	*Receptor Protein Tyrosine Phosphatases (RPTPs)/DLAR*	*Guidance and Targeting*	*Desai et al., 1997; Clandinin et al., 2001*
Neurotrophins			
NGF BDNF NT-3 NT-4	*Trk, p75*	*Chemoattractant & Growth Cone Steering; Chemorepellant w/ ↓cAMP; Modulators of other guidance molecules (Sema3A)*	*Gallo et al., 1997; Ming et al., 1999; Song et al., 1997; Dontchev & Letourneau, 2002; Polleux et al., 2002*
Myelin-Associated Inhibitors *(Nogo, MAG, OMgp)*	*Nogo receptor (NgR); p75; gangliosides (GT1b)*	*Inhibition of Growth in Adult Neurons*	*Qiu et al., 2000; Chen et al., 2000; Wang et al., 2002*

leech CNS (McKay et al., 1983). Axon fasciculation is a pathfinding strategy utilized by the neurite as it grows out toward its target. Often axon bundles (fascicles) will initially extend in the same direction based on 'nerve tracts' that are formed by 'pioneer cells' (i.e., the first neuron in the bundle). These axon bundles will then grow out along the nerve tracts until they reach a choice point where they must decide to defasciculate and navigate toward their final target (Brummendorf and Rathjen, 1997). Further analysis of Lan 3-2 revealed that it was a mannosidic epitope (McKay et al., 1983; Flanagan et al., 1986; Bajt et al.,, 1990) and that it was necessary for the successful defasciculation and arborization of developing sensory afferents after they reached the CNS (Zipser et al., 1989; Zipser and Cole 1991; Song and Zipser, 1995). Thus, Lan 3-2 is a necessary component of axon pathfinding and target selection in leech during regeneration and development. Moreover, the Lan 3-2 positive proteins were later identified as two novel cell adhesion molecules, LeechCAM and Tractin (Huang et al., 1997; Jie et al., 1999; Jie et al., 2000). In this study, the Lan 3-2 antibody has been utilized to investigate the molecular correlates of neural morphallaxis (Chapter II, III, IV, and V). These studies indicate that neural morphallaxis in *Lumbriculus variegatus* may involve molecular signaling events similar to those involved in embryonic neural development in annelids.

Objectives of This Study

> Worms have played a more important part in the history of the world than most persons would at first suppose.
> - Charles Darwin, The formation of vegetable mould through the action of worms with observations on their habits.

In 1990, Drewes and Fourtner described regeneration within the nervous system of an oligochaete worm, *Lumbriculus variegatus*. They demonstrated that the functional organization of escape reflexes was highly plastic during morphallactic regeneration following body fragmentation and speculated that this plasticity may result from the counterbalance of morphogenic influences localized within the anterior and posterior ends of regenerating body fragments. Thus, the focus of this study was to characterize cellular and molecular mechanisms that regulate the reorganization of neuronal pathways underlying rapid escape reflexes of *Lumbriculus variegatus*, specifically during morphallaxis. To that end, a general hypothesis of this study is that the cellular and molecular correlates of neural morphallaxis likely involve mechanisms comparable to other forms of neural plasticity including those described during development and learning and memory.

Specific Aims of Chapters

Chapter II

Neural morphallaxis is marked by changes in giant fiber anatomy and physiology. It is likely that a morphogenic signal, arising from the idiomorphically regenerating head or tail, may instruct these cellular changes. Experiments using boric acid blockade of epimorphic regeneration, demonstrate that regenerating head and tail

segments do not affect neural morphallaxis. Additionally, neural morphallaxis is examined in asexually reproducing worms, where it exhibits a different temporal pattern of morphallactic changes.

Chapter III

Axonal regeneration in *Lumbriculus* likely involves the up- or down-regulation of proteins which are known to be involved during axonal outgrowth. Because many of the anatomical and physiological changes associated with neural morphallaxis are found within the giant fiber system of the animal, I've hypothesized that known axonal markers may also be differentially expressed. Experiments using the leech antibody, Lan 3-2, provide the first evidence of the molecular changes associated with neural morphallaxis in *Lumbriculus*.

Chapter IV

Studies of regeneration in other invertebrate systems implicate the importance of nerve injury 'signals' for successful (and functional) regeneration. Thus, it is likely that damage to the nervous system may result in the release of signals which may play an important role during neural morphallaxis in *Lumbriculus*. Experiments using either dorsal amputation (no nerve cord damage) or ventral amputation (nerve cord damage) of animals demonstrate that damage to the nerve cord is necessary for induction of neural morphallaxis but, not sufficient to maintain these changes.

Chapter V

It is thought that regeneration likely involves the differential expression of many genes or proteins known to play important roles during development or other forms of plasticity. It is likely that neural morphallaxis, also involves similar molecular changes. Protein profiles of morphallactic worm tissue were examined during both epimorphic regeneration and asexual reproduction. Additionally, mass spectrometric analysis was used to possibly identify the proteins of interest.

CHAPTER II

ASEXUAL REPRODUCTION AND SEGMENTAL REGENERATION, BUT NOT MORPHALLAXIS, ARE INHIBITED BY BORIC ACID IN *Lumbriculus variegatus* (ANNELIDA: CLITELLATA: LUMBRICULIDAE)*

Overview

Body fragmentation, in some animal groups, is a mechanism for survival and asexual reproduction. *Lumbriculus variegatus* (Müller, 1774), an aquatic oligochaete worm, is capable of regenerating into complete individuals from small body fragments following injury and reproduces primarily by asexual reproduction. Few studies have determined the cellular mechanisms that underlie fragmentation, either regenerative or asexual. We utilized boric acid treatment, which blocks regeneration of segments in amputated fragments and blocks architomic fission during asexual reproduction, to investigate mechanistic relationships and differences between these two modes of development. Neural morphallaxis, involving changes in sensory fields and giant fiber conduction, was detected in amputated fragments in the absence of segmental regeneration. Furthermore, neural morphallactic changes occurred as a result of developmental mechanisms of asexual reproduction, even when architomy was prevented. These results show that fragmentation in *L. variegatus*, during injury or asexual reproduction, employs developmental and morphallactic processes that can be

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* This work has been accepted for publication in Hydrobiologia.

mechanistically dissociated by boric acid exposure. In regeneration following injury, compensatory morphallaxis occurred in response to fragmentation. In contrast, anticipatory morphallaxis was induced in preparation for fragmentation during asexual reproduction. Thus, various forms of regeneration in this lumbriculid worm can be activated independently and in different developmental contexts.

Introduction

The freshwater oligochaete worm, *Lumbriculus variegatus* (Müller, 1774), possesses a remarkable capacity for the regeneration of lost body parts and reproduces asexually by architomy. These developmental processes involve two general patterns of regeneration: epimorphosis and morphallaxis. Epimorphic regeneration is characterized by the differentiation of a blastema and the *de novo* generation of body parts or tissues; in this case, the regeneration of head or tail body segments. Morphallaxis, on the other hand, is a pattern of regeneration that involves the transformation of existing body parts or tissues into newly organized structures. This reorganization of extant tissues does not require the proliferation of new cells. During oligochaete morphallaxis, original body segments undergo anatomical and physiological transformations to match their new positional identity along the animal's body axis (Drewes and Fourtner, 1990; Myohara et al., 1999). Since a limited number of head segments are regenerated in the lumbriculid, *L. variegatus*, and the enchytraeid, *Enchytraeus japonensis*, posterior segments often become relocated anteriorly, requiring dramatic changes in the anatomy and physiology of these segments as they change positional identity.

Drewes and Fourtner (1990) demonstrated the functional reorganization of escape reflexes and their neural correlates in *L. variegatus* during morphallactic regeneration. Rapid escape behaviors in this worm are mediated by specific giant fiber pathways. A conserved feature of virtually all oligochaetes is the presence of three giant fibers, located in the dorsal region of the ventral nerve cord, that mediate rapid escape reflexes (Zoran and Drewes, 1987). These through-conducting pathways have been described as a chain of segmentally-arranged giant axons that run the length of the nerve cord (Günther and Walther, 1971). The medial giant fiber (MGF) mediates head specific escape behaviors such as rapid anterior shortening and body reversal, while the paired lateral giant fibers (LGF) govern tail withdrawal and activation of swimming bouts (Drewes and Fourtner, 1989; Drewes, 1999). Morphallaxis of these neural pathways during changes in segmental position, as in hydra regeneration (Wolpert et al., 1971), may result from the counterbalance of morphogenic influences localized within anterior and posterior activation centers at the ends of regenerating body fragments.

Recent studies have characterized several paradigms for the investigation of cellular and molecular events associated with morphallaxis (Martinez and Zoran, 2000; Lesiuk and Drewes, 2001a,b). Specifically, body segments isolated from posterior regions, which acquire a new, more anterior segmental position, exhibit transformations in touch sensory fields, giant fiber conduction velocity, axonal diameter, and other physiological properties appropriate for the fragment's new positional identity. Lesiuk and Drewes (2001b) demonstrated the formation of ectopic heads in *L. variegatus* as a result of ventral nerve cord ablation. Following ectopic head formation, morphallactic

changes occur within the giant fiber system, but only in 10-15 segments posterior to the newly formed ectopic head. These studies suggest that formation of a new head, via epimorphosis, may be required for activation of developmental mechanisms that regulate (neural) morphallaxis.

Boric acid, the most prevalent form of environmental boron, has been shown to be toxic in many species through its impact on reproductive and developmental events (Shomron and Ast, 2003), though its actions are not thought to be carcinogenic or mutagenic (Moore, 1997). It has been suggested that boric acid might disrupt epimorphosis (segmental regeneration) in lumbriculid worms (Drewes, 2003). Here we utilized boric acid treatment to test whether disruption of segmental regeneration would abrogate mechanisms of neural morphallaxis in *L. variegatus* (Martinez and Zoran, 2000). In addition, we have set out to test whether morphallaxis is dependent on concomitant epimorphosis, employing boric acid to disrupt epimorphic production of new head and tail buds. We demonstrate that fragments do not undergo head or tail formation in the presence of active concentrations of boric acid. Nonetheless, morphallaxis was still detected in these fragments, suggesting that morphallactic mechanisms are not dependent on ongoing epimorphosis. Furthermore, boric acid suppressed architomic fission in worms, but did not disrupt morphallaxis. Therefore, developmental mechanisms requiring morphallaxis are independent of the processes governing segment formation (epimorphosis) and architomy.

Materials and Methods

Animals and Maintenance

Lumbriculus variegatus were purchased from Flinn Scientific, Inc. (Batavia, IL). They were housed in moderately aerated Ozarka spring water, at a constant temperature of about 16ºC in the dark. Worms were provided brown paper towel clippings for substrate and were fed *Spirulina* powder and Tetramin staple flakes twice weekly.

Generation of Regenerating Body Fragments

To obtain experimental body fragments, worms were briefly anesthetized in 0.25 mM Nicotine in spring water. Segmental amputations were made at intersegmental boundaries with microdissecting scissors. Body fragments consisted of approximately 30 segments from either the anterior third or the posterior third of the worm. Regenerating body fragments were maintained individually in containers of spring water at 16ºC. Although animals regularly fragment by asexual fission in laboratory cultures, the rate of fission is depressed in animals maintained with sufficient aeration and substrate at cool temperatures. To induce production of body fragments by asexual fission, worms were exposed to an environmental shift. Animals were moved to room temperature (22ºC) for 3-4 days and then returned to 16ºC culture conditions that lacked paper substrate and aeration. Following this shift in environmental quality, ninety percent of animals typically fragment in 3-4 weeks.

Boric Acid Treatment

Whole animals, or body fragments, were immersed in spring water containing boric acid (99.9% purity; 100 pM-50 mM) for 2 weeks. Animal survival and segmental regeneration was monitored daily. Newly regenerated head and tail buds were visually inspected for the presence of defined segmental characteristics such as segmental boundaries and organization of the vasculature. Segments were counted as true segments based on the presence of defined intersegmental boundaries. Measurements of fragment weight, sensory fields, and giant fiber conduction velocities were calculated weekly. At the conclusion of exposure and testing periods, animals were dissected and fixed for histological analysis.

Electrophysiological Recording

Impulse conduction along giant nerve fibers was studied using non-invasive electrophysiological recordings (O'Gara et al., 1982; Drewes and Fourtner, 1990). Touch stimuli were delivered by a hand-held plastic probe. Medial giant fiber (MGF) and lateral giant fiber (LGF) action potential waveforms were distinguished based on previously reported spike characteristics (Drewes and Fourtner, 1989, 1990; Rogge and Drewes, 1993). Extracellular voltage recordings were obtained using a printed-circuit-board grid of electrode pairs (1 mm space between positive and negative electrodes) and electrical signals were preamplified using a pair of differential recording amplifiers (100x gain, AC-coupled differential inputs). These spike recordings were digitized with a Powerlab A-D conversion system (ADInstruments, Inc.) and were analyzed on a G4 Macintosh computer (Apple, Inc.) using the Powerlab Chart software.

Spike conduction time between two pairs of recording electrodes (5 mm distance between the electrode pairs) was measured from peak-to-peak of giant fiber spike waveforms recorded on the corresponding input channels. Conduction velocity (m/s) was obtained by dividing the conduction distance by the spike conduction time. Individual means, based on 3-5 measurements per animal, were used in calculating group means. Non-invasive recording grids were also used to map giant fiber sensory fields. Segments of specific identity (e.g., segment number 50) were marked with a spot of water-insoluble ink (Sharpie). Individual segments were then touched with a probe and giant fiber responses were monitored electrophysiologically.

Histology

Whole mount preparations were first pinned out on sylgard blocks while immersed in 0.25 mM nicotine in spring water, which blocks reflexive muscle movements and prevents autotomy (Lesiuk and Drewes, 1999). The VNC was exposed by removing the digestive tract, nephridia, and ventral blood vessel. Fragments were fixed in 4% paraformaldehyde, washed in PBS, stained with a dilute solution of toludine blue, rinsed with water and mounted on slides for light microscopy. All fixative and buffer solutions were maintained at $\pm 4^{o}$C and at pH 7.2.

Preparations were imaged using an Olympus inverted microscope, DIC optics, and a Hamamatsu CCD camera. Images were captured with Simple PCI software (C-Imaging, Inc.) and imported into Adobe Photoshop 6.0. Image analysis was conducted to select equivalent regions of interest from MGF and LGF dorso-longitudinal profiles along the length of the ventral nerve cord. GF diameters were measured for both MGF

and LGF from whole mount preparations. Multiple measurements were made from each preparation along the length of a fragment and group means were then calculated.

Statistics

Two-tailed student's t-tests (Microsoft Excel) or ANOVA (Statistica, Inc.) were used for statistical analysis. Data are presented as mean plus or minus standard deviation (s.d.) or standard error of the mean (s.e.m.) as indicated. Statistical significance was $p < 0.05$.

Results

Effects of Boric Acid on Segmental Regeneration

Following amputation, body fragments of 30 segments always regenerate a head of 8 segments and a tail of variable length, depending upon the fragment's original mass and positional identity (Fig. 4a). Exposure of fragments to 10 mM boric acid (BA) for 2 weeks disrupted segment formation without resulting in animal lethality (Fig. 4b). Lethality dose-response curves show that higher concentrations of BA (25-50 mM) produced significant reductions in fragment survival, while concentrations of 1 mM, 1 nM, 100 pM, and lower caused no mortality in worm fragments. However, concentrations of BA less than 10 mM also failed to disrupt segmental regeneration completely. Fragments exposed to 10 mM BA exhibited normal wound healing, but abnormal blastema formation. Consequently, initial bud formation was delayed until 4-5 days following amputation; whereas, in lower concentrations of BA, head and tail buds were visible 1-2 days post-amputation. In 10 mM BA, both anterior-third and posterior-

third fragments had severe developmental defects in the number of head and tail segments produced as compared to untreated control fragments from similar axial regions of origin (Fig. 4c-e). Fragments treated with boric acid produced head buds that were reduced in size, from 8 well-defined segments to 3-5 segments, and lacked structural organization and a distinct prostomium (Fig. 4f,g). The impact of boric acid was most pronounced in tail regions of fragments. Tail buds produced by fragments of both anterior and posterior axial origin failed to develop any well-defined segments (Fig. 4g).

Analysis of Neural Morphallaxis in the Absence of Segmental Regeneration

Giant fiber mediated pathways are activated by sensory field inputs that vary along the anterior to posterior length of the animal. In our control animals, activation of touch receptors in the anterior 1/3 region of the animal's body wall (44 ± 2.1segments, ~29% of total segments) triggered only the medial giant fiber (MGF), resulting in a head withdrawal (Fig. 5a). In the posterior 2/3 region of the worm (93.2 ± 2.5 segments, ~62% of the total segments), tactile stimulation elicited only lateral giant fiber (LGF) spiking, which resulted in a tail withdrawal. MGF and LGF sensory fields coexist in only 12.8 ± 2.3 segments of sensory field overlap, the equivalent of 9% of body segments (Fig. 5a). Therefore, fragments of 30 segments removed from anterior and posterior regions possessed exclusively MGF and LGF sensory fields, respectively, at the time of amputation. During neural morphallaxis, fragments removed from posterior regions transformed their escape reflex circuitry (i.e., gained MGF sensory activation),

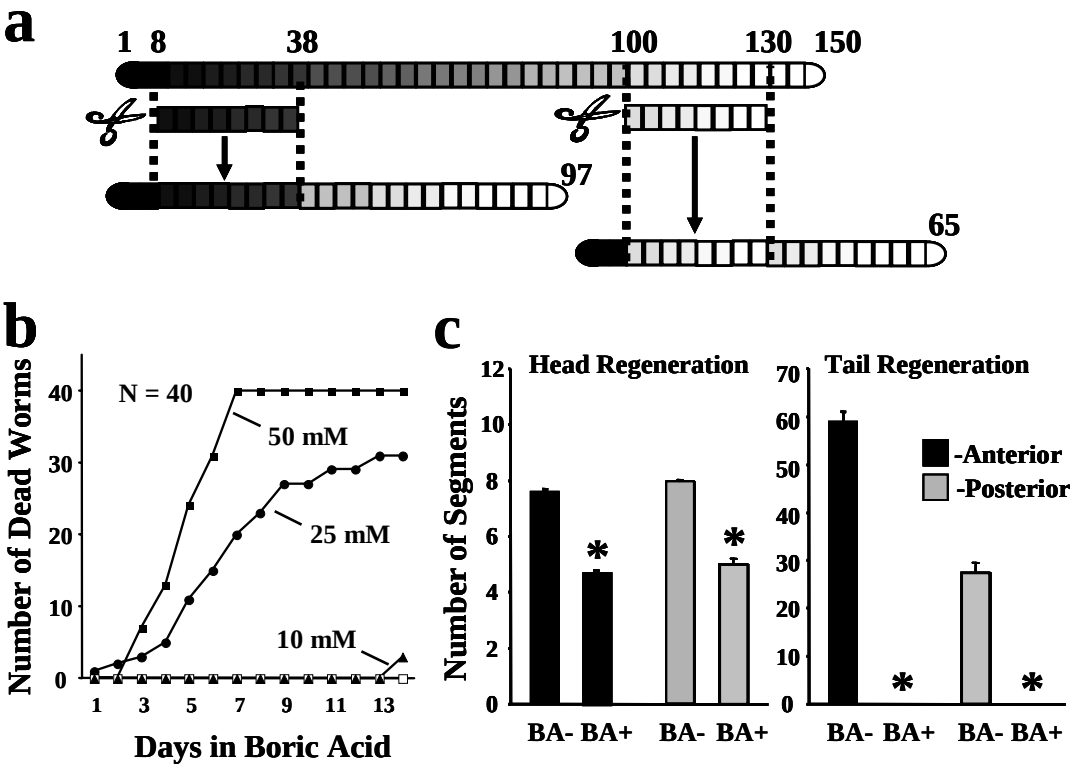

Figure 4 - Effects of boric acid on segmental regeneration.

a. Segmental regeneration in L. variegatus. Whole animals of approximately 130 segments in length were cut into pieces, there by generating two populations of fragments: one from anterior (segment ~8-38) and one from posterior (segment ~100-130) regions of the worm. Amputated fragments always regenerated 7-8 segments of head and variable lengths of tail. Posterior fragments, which originally comprised segments 100-130, became more anteriorly positioned following segmental regeneration of the short head and longer tail. Numbers represent segmental numbers and shading implies anterior-posterior diffusion in original segment positional identity.

b. Lethality dose response of boric acid. Graph showing lethality of concentrations of boric acid on L. variegatus. Concentrations ranged from 0 (open squares) to 50 mM (as indicated). Experimental number equals 40 animals per group.

c. Boric acid treatment of segmentally regenerating fragments. Head and tail regeneration in both anterior (n = 39) and posterior (n = 36) fragments was significantly reduced by 10 mM boric acid (, $p < 0.001$). Bars = group mean. Error bars give s.d.*

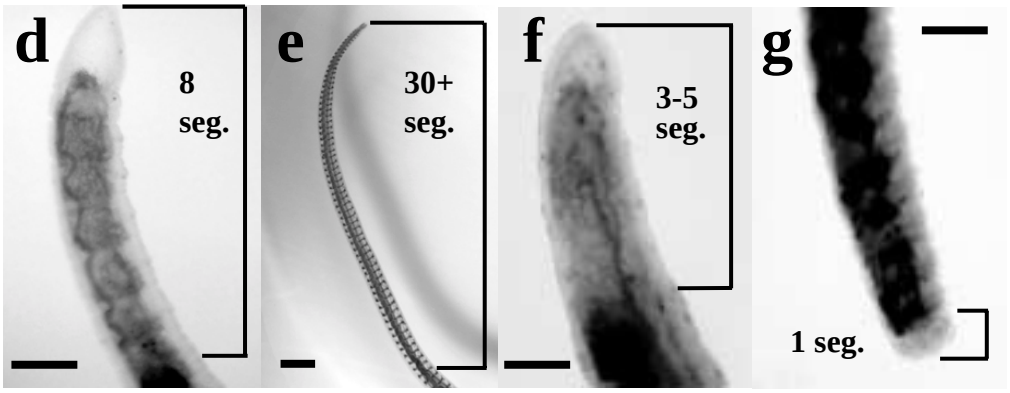

Figure 4 – (Continued).

d- g. Regenerated head and tail segments on control fragments cultured in spring water (d & e) and experimental fragments cultured in 10 mM boric acid (f & g). Although some segmental regeneration of head segments occurred during one week in BA, disorganized structure of these segments (note the abnormal vasculature) was evident (f). Tail buds, even one week after BA treatment, were virtually nonexistent. All images were captured at 3 weeks after amputation. Scale bars = 2 µm (d,f& g) and 0.6 µm (e).

thus responding with behaviors appropriate for their new (anterior) positional identity. Fragments treated in boric acid exhibited changes in giant fiber sensory fields similar to those of normal regenerating fragments (Fig. 5b). Specifically, 88% of posterior fragments treated in boric acid produced head withdrawal responses following two weeks of regeneration. Therefore, transformation of giant fiber sensory fields was not affected by BA-induced inhibition of segmental regeneration. In addition, BA-treated fragments exhibited changes in giant fiber conduction velocity that were appropriate for the fragment's new position along the length of the animal.

Prior to amputation and BA exposure, MGF and LGF spikes were propagated through the posterior-third of the worm's body with similar conduction velocities (Fig. 5c,e). Following amputation and acute exposure to BA, tactile stimulation within the posterior-third of the worm's body did not result in activation of the medial giant fiber but LGF spikes were conducted at approximately 6 m s^{-1} (Fig. 5e). However, a gradual

increase in MGF conduction velocity was detected in these fragments over a subsequent 2 weeks in BA, such that conduction velocities eventually attained values similar to those of anterior control segments. These data indicate that neural morphallaxis occurred in posterior fragments, even in the absence of segmental regeneration.

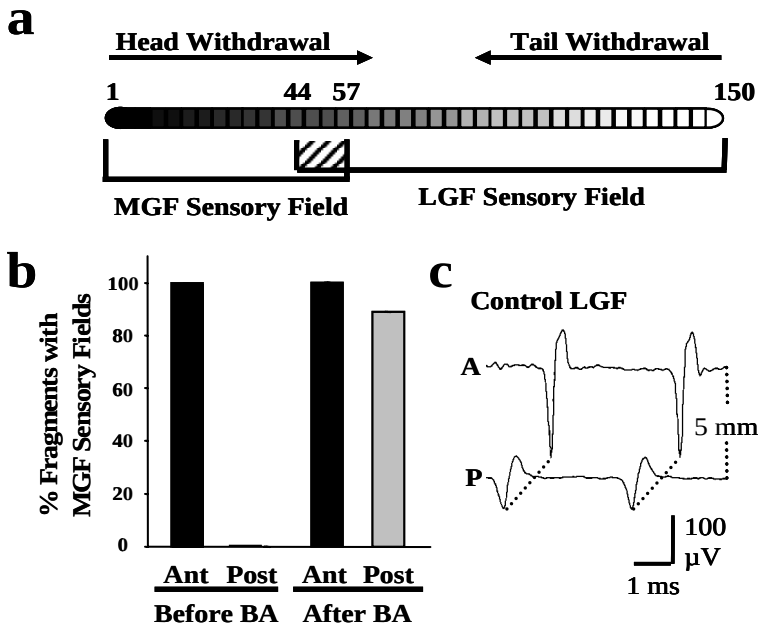

Figure 5 - Boric acid does not effect changes in sensory field or conduction velocity during neural morphallaxis.

a. Giant fiber sensory field map for control whole animals. Head and tail rapid escape withdrawal was recorded following tactile stimulation to body segments. Specifically, stimulation of the anterior 1/3 region (segment 1-43) activated only a medial giant fiber (MGF) spike resulting in a head withdrawl. Stimulation of the posterior 2/3 region (segment 58-150) activated only lateral giant fiber (LGF) spiking causing a tail withdrawal. An area where both MGF and LGF spikes were activated (sensory field overlap) was detected, on average, between segments 44-57 (n = 6).

b. MGF sensory fields in fragments before and 3 weeks after boric acid treatment. Prior to boric acid (10 mM) exposure, newly amputated anterior fragments possessed medial giant fiber sensory fields in 100% of the population (n = 34). Medial giant fiber sensory fields were never detected in posterior fragments prior to boric acid treatment (n = 39). Following BA treatment, greater than 85% of posterior fragments possessed MGF sensory activation in a portion of the original 30 segments.

c. Representative giant fiber electrophysiological recording from a control fragment. To measure conduction velocities, MGF spikes or LGF spikes (as in this example recording) were recorded from two different electrode pairs set 5 mm apart. Velocities were measured by dividing the conduction distance by the peak-to-peak conduction time (as indicated by dotted lines). A and P equal anterior and posterior recording. Thus, this LGF spike is conducting in a posterior–to–anterior direction.

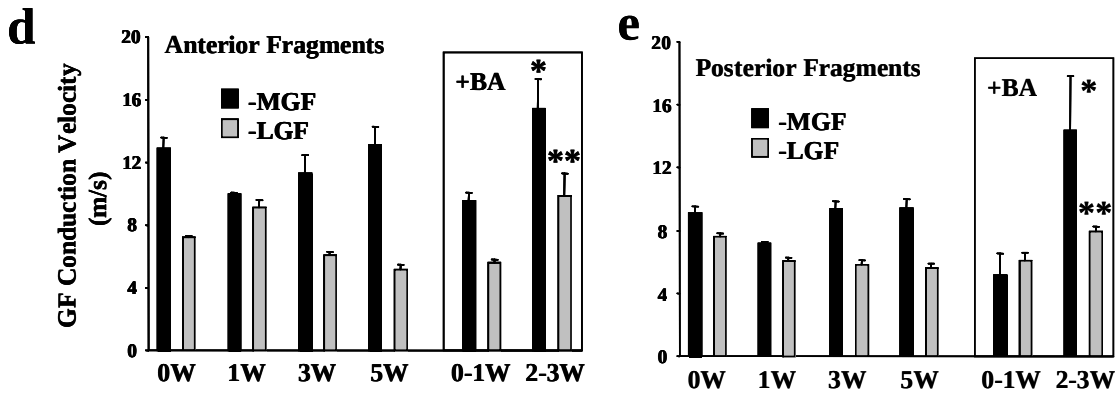

Figure 5 – (Continued).

d. Giant fiber conduction velocity in anterior fragments during segmental regeneration. Prior to amputation (0 W), MGF conduction velocities measured 12.9±0.7 m $s^{-1}$and LGF conduction velocities measured 7.2±0.1 m s^{-1}in the anterior region of the worm (n = 8). Following amputation, MGF and LGF conduction velocities had equalized to approximately 10 m s^{-1}at 1 week (1 W) and then returned to normal rates after 5 weeks of segmental regeneration. Fragments treated in boric acid (boxed region of histograms) demonstrated similar, relative changes in conduction velocity between MGFs and LGFs during segmental regeneration (n = 9), although both MGF and LGF velocities were significantly greater than controls at 3 weeks (, p < 0.05; **, p < 0.0001).*

e. Giant fiber conduction velocity in posterior fragments during segmental regeneration. Prior to amputation (0W), MGF conduction velocities measured 9.1±0.1 m $s^{-1}$and LGF conduction velocities measured 7.6±0.4 m s^{-1}in the posterior region of L. variegatus (n = 8). Following amputation, MGF conduction velocities increased while LGF velocities decreased, indicative of the fragment's new anterior position. Even when treated in boric acid, relative changes in giant fiber conduction velocity emerged in posterior fragments by 2-3 weeks post amputation (n = 8). MGF and LGF conduction velocity in BA treated fragments were significantly greater than those of control fragments at 3 weeks (, p < 0.05; **, p < 0.0001). Bars = group mean. Error bars = s.d.*

The diameters of the three giant fibers (Fig. 6a) vary along the length of the ventral nerve cord. MGFs have diameters that are much larger in the anterior region of the worm and then taper in size posteriorly. MGF and LGF diameter in posterior fragments was significantly changed following 9 weeks of morphallaxis (Fig. 6b). GF diameters in anterior fragments remained relatively unchanged during regeneration. Fragments treated with 10 mM BA, both anterior and posterior, exhibited no significant differences in GF diameter, as compared to untreated control fragments. Thus, morphallactic changes in giant fiber size were not impacted by BA exposure.

Asexual Reproduction in Lumbriculus variegatus

Many oligochaetes, including *L.* variegatus, have marked regenerative capabilities and reproduce by asexual fission. Since boric acid blocked segmental regeneration without affecting neural morphallaxis, we tested the hypothesis that asexual

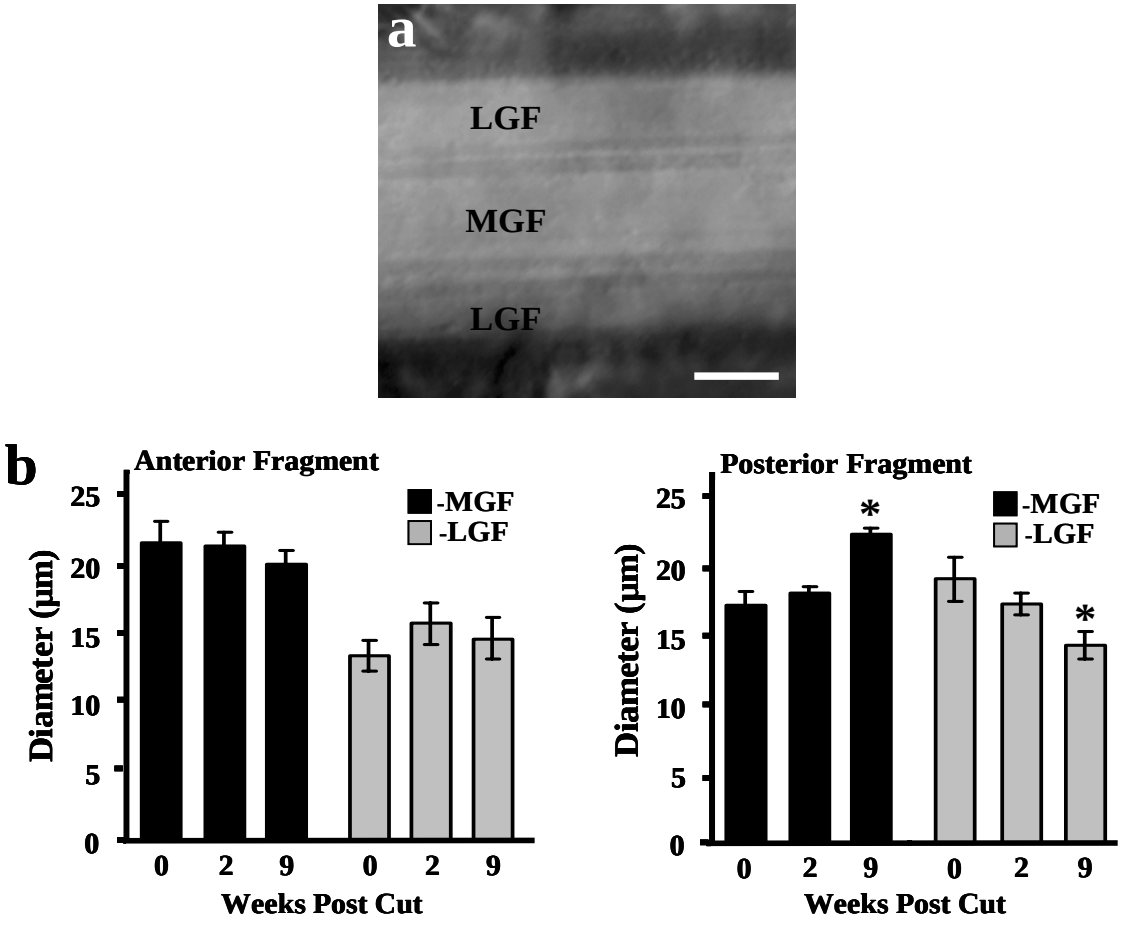

Figure 6 - Boric acid does not effect changes in giant fiber size during neural morphallaxis.

a. Photomicrograph of an anterior fragment using Nomarski optics. Using similar images collected for all experimental groups, giant fiber diameters were measured by selection of equivalent regions of interest (using SimplePCI software) from giant fiber dorsal-longitudinal profiles along the length of the nerve cord. Axonal diameters were measured for both LGF and MGF in regions of interest. Scale bar = 15 µm.

b. Changes in giant fiber diameter during neural morphallaxis. No significant change in giant fiber diameter was detected in anterior fragments following 0, 2, and 9 weeks of regeneration. However, posterior fragments showed a marked decrease in LGF diameter after 9 weeks of regeneration ($p < 0.02$, $n = 9$). Significant changes in MGF size were also detected in posterior fragments after 9 weeks of regeneration ($p < 0.002$, $n = 9$).

fission is mediated by a developmental mechanism that can be dissociated from processes underlying morphallactic regeneration. To test this hypothesis, we developed an environmental shift paradigm (see Methods) to induce asexual reproduction by fragmentation.

Following environmental shift, controls animals in three separate experiments (involving a total of 76 worms) fragmented at a rate of 55% of the population over a 3-week period. In contrast, no worms fragmented in parallel control cultures treated with 10 mM BA, even though they had experienced the environmental shift protocol (Fig. 7a). Thus, BA inhibits asexual reproduction in this *L. variegatus* paradigm. Asexually reproducing lumbriculid worms formed an initial zone of architomy (Fig. 7b) that was condensed over several days into an architomy site (Fig. 7c).

Fragmentation by asexual reproduction resulted in the generation of two, zooid fragments. Interestingly, we never observed more than two fragments produced by any one animal using this protocol. Furthermore, the architomic fission site was predictable. Fragmentation resulted in the production of an anterior fragment of 48$\pm$10 segments (mean$\pm$s.d.) and a posterior fragment of 92$\pm$14 segments (Fig. 7d). The two zooids produced by asexual fission in these experiments each formed a blastema at the site of fragmentation, which then differentiated appropriately into head or tail buds. Additionally, wet-weight measurements were collected for each fragment produced via asexual fragmentation. Anterior clones, on the day of fragmentation, measured 5.7$\pm$1.6 mg (mean$\pm$s.d.) and posterior clones measured 5.0$\pm$1.0 mg (n = 23; Fig. 7e). Because

there is no significant difference between the masses of anterior and posterior clones, we conclude that asexual fragmentation, also generating two fragments of significantly

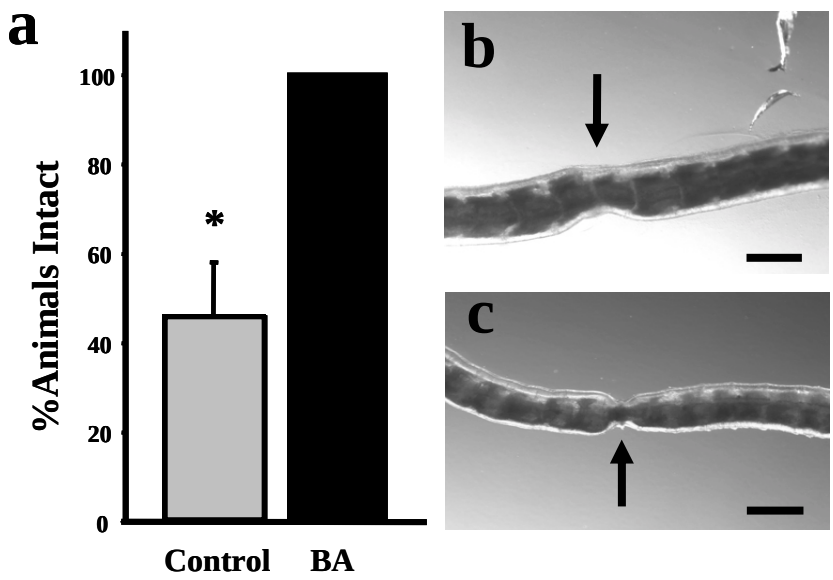

Figure 7 - Asexual reproduction in Lumbriculus variegatus.

a. Asexual reproduction was induced in whole animals by an environmental shift in culture conditions (see methods). Fragmentation, and consequently asexual reproduction, was blocked in animals treated with boric acid (10 mM). Following three weeks (two of these weeks being in BA-treated spring water), 100% of the animals had not fragmented or produced autotomy sites (n = 76). In control populations, 50% of animals fragmented by week 3 (n = 76).

b & c. L. variegatus reproduces asexually through architomic fission. During asexual reproduction, fission zones are formed (arrow in b, early fission). Obvious autotomy sites (arrow in c, late fission) are present prior to fission. No new segmental differentiation was seen in segments adjacent to the fission site, a feature characteristic of architomy. Scale bars = 2 µm.

different segment numbers ($p < 0.0001$), resulted in the production of clones of equitable mass ($p = 0.1004$).

While neural morphallaxis has been described in fragments that were produced via injury, its involvement during asexual fragmentation has not been investigated. Our combined studies of giant fiber sensory fields and emerging architomy sites during asexual reproduction demonstrated that, on average, the predictable fission (architomy)

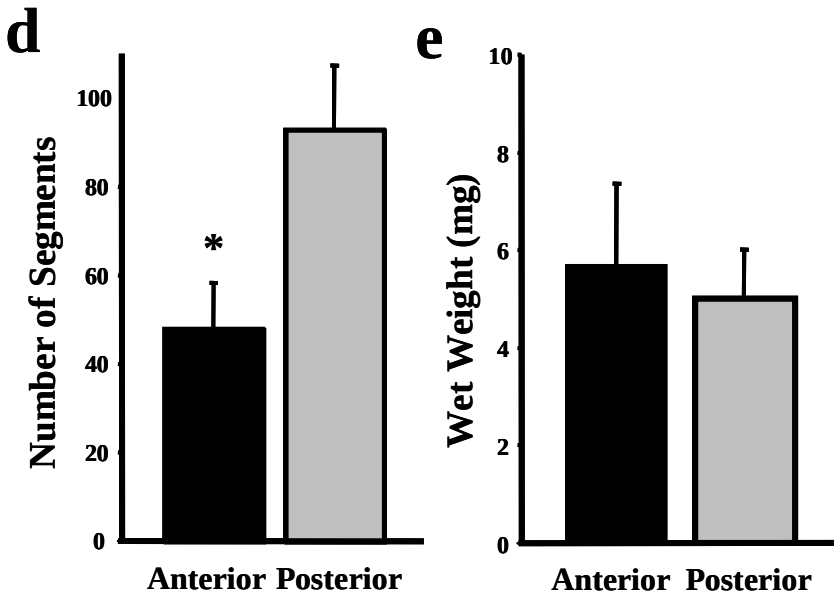

Figure 7 – (Continued).

d. Architomy sites are predictable in L. variegatus. In each animal fragmented during asexual reproduction, the numbers of head segments produced following fission were significantly less than tail segments. Architomy sites were consistently produced at segment 48±10 in animals of approximately 140 segments ($p < 0.0001$; $n = 37$).

e. Cloned fragments produced following fission were of equal mass. Anterior fragments possessed wet weights that were not significantly different ($p = 0.11$, $n = 22$) from posterior fragments, even though they possessed approximately 1/3 of the total segments of the whole animal prior to fission.

site was located virtually at the center of the zone of MGF/LGF sensory field overlap. In some cases where animals had regenerated more recently, as suggested by differences in body wall pigmentation, new fission sites were located within 2-3 segments of the previous site. As the fission site developed, sensory field mapping detected a significant expansion of this area of sensory field overlap. Specifically, an increase in the MGF sensory field was observed, largely in segments posterior to the fission site. The zone of sensory field overlap expanded from 12±2 segments (mean±s.d.) to 29±8 segments following fission site formation (Fig. 8a). Furthermore, an area of exclusively MGF

sensory field of 10-15 segments just adjacent and posterior to the architomy site was formed.

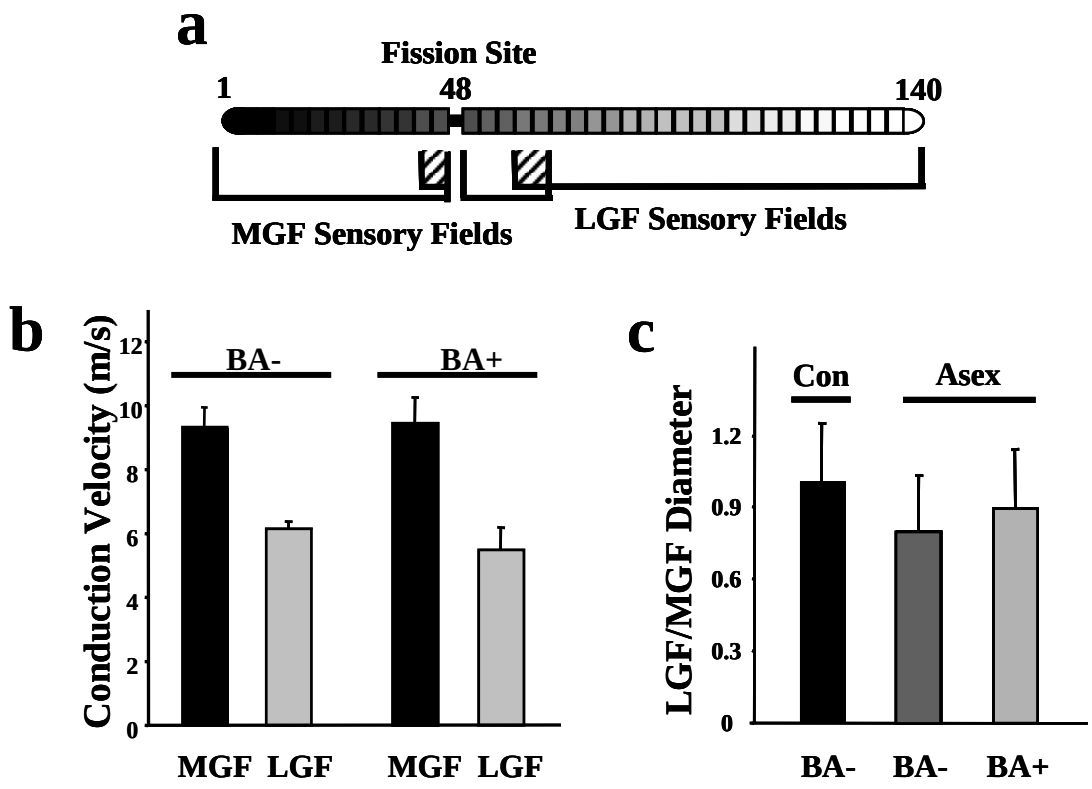

Figure 8 - Boric acid does not affect neural morphallaxis during asexual reproduction.

a. Giant fiber sensory fields expanded prior to fragmentation during asexual reproduction. Fission sites developed at approximately segment 48, which was within the zone of MGF/LGF sensory field overlap in these animals. Sensory field mapping of asexually reproducing animals (w/ fission sites) demonstrated an increase in the MGF sensory field in segments posterior to the fission site. Sensory field overlap expanded from 12 segments in controls to 29 segments in animals exposed to the environmental shift protocol for induction of asexual reproduction. In 10-15 segments posterior to the autotomy site, only MGF spikes could be evoked by tactile stimulation (n = 6).

b. Boric acid did not affect changes in giant fiber conduction velocity during asexual reproduction. MGF and LGF conduction velocities, measured within nascent or extant fission zones in both boric acid treated and control animals respectively (n = 5 per group) where not significantly different.

c. Changes in giant fiber diameter ratio were not effected by boric acid during asexual reproduction. There was no significant difference between giant fiber diameter ratios of animals treated with boric acid or spring water (n = 5 per group), following induction of asexual reproduction (Asex). Giant fiber diameter changes where comparable to those measured in control animals, which were not asexually reproducing (Con).

Asexual Fragmentation Is Blocked by Boric Acid with No Effect on Neural Morphallaxis

To test the effects of boric acid on neural morphallaxis during asexual reproduction, we recorded morphallactic changes in giant fiber conduction velocity and axonal diameter following BA treatment. We measured conduction velocities specifically within segments 40 and 70 in asexually fragmenting (BA-) and non-fragmenting (BA+) animals. This region represented the segments with expanding MGF sensory fields, that is, segments posterior to the nascent fission site. Medial giant fiber conduction velocity recordings from BA-treated animals measured 9.5±0.8 m s^{-1} (mean±s.d.), as compared to MGF velocities of 9.3±0.6 m s^{-1} in non-fragmenting (BA-) animals (Fig. 8b). Thus, boric acid inhibition of fragmentation during asexual reproduction did not significantly effect alterations in GF conduction velocity associated with neural morphallaxis. Giant fiber size (axonal diameter) in asexually regenerating animal was not significantly different than that of animals treated with boric acid (Fig. 8c), again suggesting that the cellular correlates of neural morphallaxis are resistant to disruption by boric acid. Additionally, these GF size relationships did not differ from untreated control animals (not reproducing asexually). Taken together, these results suggest that neural changes associated with morphallactic regeneration occurred in an anticipatory fashion (prior to architomy) during asexual reproduction.

Discussion

Asexual reproduction and regeneration of lost segments are found in several groups of annelid worms, including several families of oligochaetes (Brinkhurst and Jamieson, 1971). Those oligochaetes that reproduce asexually typically possess the

ability to regenerate head and tail segments following body transection (Morgulis, 1907; Berrill, 1952; Christensen, 1959; Myohara et al., 1999; Bely and Wray, 2001), with some exceptions (Bely, 1999). The ability of some oligochaetes, but not others, to regenerate body fragments, may be a consequence of the environmental niches in which they have evolved (Drewes and Zoran, 1989; Bely 1999). *Lumbriculus variegatus* lives in shallow margins of ponds, where rapid environmental change and frequent predatory attack may be common. Asexual reproduction by architomic fission and regeneration following body injury are both common aspects of the life history of *L. variegatus* (Fig. 9a). These annelids are also hermaphrodites capable of reproducing sexually, however, they reproduced only by asexual reproduction in normal laboratory conditions. Asexual reproduction in *L. variegatus*, cultured under controlled environmental conditions, involves a predictable fission site and reproductive outcome. Because fragmentation occurs prior to significant differentiation of head or tail buds, we conclude that fission in *L. variegatus* was by architomy. This form of asexual fission is rare among oligochaetes, where paratomic fission is the more common mode of reproductive fragmentation in those groups that have been extensively studied (Drewes and Fourtner, 1991; Bely, 1999; Bely and Wray, 2001). In paratomy, substantial head and tail segmental regeneration occurs prior to the actual separation of fragments (Giese and Pearse, 1975). The process of bud formation following architomic fission was much slower than bud formation following injury and wound healing (segmental regeneration). Buds with readily visible segmental boundaries exist at 3-4 days following body transection, while such buds are not evident on fragments produced by asexual fission

until 8-11 days post-fragmentation. The delay in transition from blastema to bud in asexual reproduction may be slowed by the need for dedifferentiation of body wall tissue covering the initial blastema of asexual buds. Architomy sites formed at the anterior-to-posterior, one-third/two-third segmental boundary. This highly predictable location suggests that developmental mechanisms work to predetermine the site of asexual fission in this species. In contrast, in other annelids, for example, some enchytraeids, each asexual reproduction event leads to the generation of 5-10 fragments from a single animal (Yoshida-Noro et al., 2000).

Both asexual reproduction and segmental regeneration in *L. variegatus* utilize blastema formation and epimorphosis to add additional body segments to the fragment. A similar process exists in *Enchytraeus japonensis* (Myohara et al., 1999). This developmental process of epimorphosis is a form of compensatory regeneration found in oligochaetes. Another example of compensatory regeneration occurs in the tubificid worm, *Branchiura sowerbyi*, where gill filaments are produced on adult mid-body segments following loss of the gill filaments specific to posterior segments in adult worms (Drewes and Zoran, 1989). Both asexual fission and regeneration in *L. variegatus* involve morphallaxis, where adult segments transform the fragment to match their new positional identity. Previous studies characterized physiological and anatomical changes that occur in the lumbriculid central nervous system during neural morphallaxis (Drewes and Fourtner, 1990). Since fragments regenerate only a small number of new head segments, the anterior most segment of a posterior fragment becomes more anteriorly positioned following segmental regeneration. Since sensory systems and escape

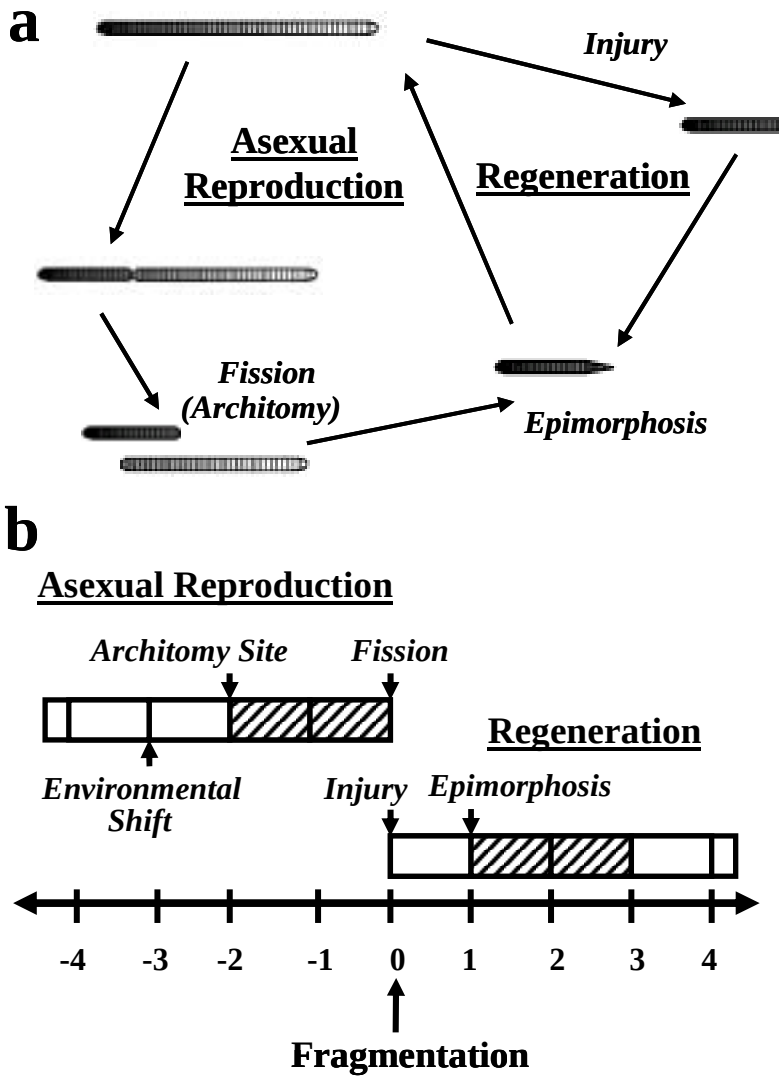

Figure 9 - Asexual reproduction and segmental regeneration involve different sequences of developmental events.

a. Lumbriculus variegatus generated new individuals from fragments of body segments by asexual reproduction and regeneration. During asexual reproduction, whole animals produce architomic fission sites and following fragmentation the original animal separates into two body fragments. The formation of new segments by epimorphosis (bud formation) leads to the development of a complete adult worm. During regeneration following injury (or experimental transection), fragments form new buds by epimorphosis at the transection site.

b. Neural morphallaxis (changes in giant fiber sensory fields, area, and conduction velocity) occurs during both asexual reproduction and regeneration, however, the order of regenerative events is arranged differently over time during these two processes. Changes associated with neural morphallactic regeneration (filled squares) were detected prior to fragmentation events during asexual reproduction, appearing 2 weeks before fission. During regeneration, in contrast, morphallactic changes were not detectable until 1-2 weeks after injury. Thus, morphallaxis of the neural anatomy and physiology of original fragment segments were produced by anticipatory regenerative mechanisms in asexual reproduction; whereas, morphallaxis was retroactive during regeneration induced by injury.

behaviors are mediated by mechanisms that vary along the anterior-posterior axis (Drewes and Fourtner, 1989; Drewes, 1999), neural morphallactic changes are especially dramatic in *L. variegatus*.

Many oligochaetes that reproduce by asexual fission also have significant regenerative capabilities. For this reason, it has been suggested that regenerative mechanisms might play an essential role during asexual fragmentation and may have contributed to the evolution of reproductive mechanisms such as paratomic fission (Bely, 1999; Bely and Wray, 2001).We have demonstrated that the developmental processes of segmental regeneration and architomic fission both involve neural morphallactic regeneration. Additionally, the induction of epimorphosis and fission were compromised by boric acid treatment, but the developmental mechanisms that initiated and maintained morphallaxis were spared. Although we cannot determine whether the regenerative process of morphallaxis has been co-opted for asexual reproduction from segmental regeneration, it is shared, yet separable from each.

In the absence of regenerative epimorphosis or reproductive fragmentation, significant neural morphallaxis persisted. These results indicate that at least one regenerative process, neural morphallaxis, has been incorporated in asexual reproduction and although the mechanism of action of boric acid in these studies is unknown, it has been implicated in developmental defects in other systems including vertebrate axial development (Price et al., 1996; Fort et al., 1998). Boric acid is not metabolized in humans or animals (Murray, 1995) and at low consumption is reported to aid in wound healing possibly by increasing RNA synthesis of tumor necrosis factors (Benderdour et

al., 1998), vascular endothelial growth factors and transforming growth factors (Dzondo-Gadet et al., 2002). Whether or not environmental levels of boric acid ever attain concentrations capable of impacting annelid asexual reproduction remains to be studied.

Anatomical and physiological correlates of neural morphallaxis in fragments produced by transection and asexual fission demonstrate that this form of regeneration is induced at different times relative to segmental regeneration by epimorphosis (Fig. 9b). In asexual reproduction, morphallactic regeneration is recruited in anticipation of architomy one week before the initiation of epimorphosis. In contrast, following injury-induced transection, morphallaxis occurred after the onset of epimorphosis. Thus, worms reproducing by asexual fission initiate developmental mechanisms in appropriate body regions (anticipatory morphallactic regeneration) in preparation for the future fragmentation event. Following fragmentation by injury, worms compensate for ongoing changes in axial position (compensatory morphallactic regeneration) coincident with the addition of new segments by epimorphosis. This process of anticipatory morphallaxis may not be peculiar to architomic fission, since changes is neural function likely occur prior to paratomic fission in the naidid worm, *Dero digitata*, even though the expression of these changes is inhibited prior to the fragmentation event (Drewes and Fourtner, 1991).

Not all annelids capable of segmental regeneration undergo asexual fission (Berrill, 1952), even though many of the developmental events that govern them are similar, if not co-opted. The processes of anticipatory and compensatory morphallaxis are critical elements of the developmental programs that regulate asexual reproduction

and regeneration in *L. variegatus*. Still, the cellular and molecular mechanisms that govern morphallaxis are unclear. Based on similarities to developmental mechanisms in other invertebrates (Wolpert, 1971; Struhl, 1989), it has been hypothesized that formation of head and tail activation centers during these processes may regulate the establishment of axial gradients in GF sensory fields and escape behaviors (Drewes and Fourtner, 1990). If this is the case in *L. variegatus*, these organizing centers must be competent to form in the absence of ongoing segmental regeneration, as they occur in fragments where epimorphosis has been inhibited and during asexual reproduction prior to architomy. Nonetheless, we have demonstrated that the segmental zone of MGF/LGF sensory field overlap is virtually identical to that of the zone of fragmentation during asexual reproduction. It is likely that the developmental programs that establish sensory field axial gradients are the same events that direct the locus for architomic fission.

Clearly fragmentation in oligochaetes need not be located at zones of MGF/LGF sensory field overlap. Examples of fragmentation outside this region are occasionally observed in our *L. variegatus* cultures. In *Dero*, paratomic fission sites are typically located in segments at mid-LGF sensory field levels (Drewes and Fourtner, 1991). Whether or not these differences represent systematic disparity between architomy and paratomy remains to be determined. One common aspect of the regeneration of naidid (Drewes and Fourtner, 1991), lumbriculid (Lesiuk and Drewes, 2001b), and enchytraeid worms (Yoshida-Noro et al., 2000) is that the central nervous system likely plays a mechanistic role in this developmental process. The formation of a regeneration blastema often requires ongoing nerve fiber outgrowth in annelids (Dinsmore and

Mescher, 1998). Our studies of morphallaxis during asexual fission and regeneration in *L. variegatus* provide new tools for addressing aspects of the relationship between these two developmental events and the potential role of neural influences. However, insight into the co-option of developmental mechanisms, the role of the nervous system in these processes, and the evolution of these events will not be clear until the genes that regulate them and their molecular evolutionary relationship are determined.

CHAPTER III

REGENERATION AND ASEXUAL REPRODUCTION SHARE COMMON MOLECULAR CHANGES: UPREGULATION OF A NEURAL GLYCOEPITOPE DURING MORPHALLAXIS IN *Lumbriculus*[*]

Overview

Neural morphallaxis is a regenerative process characterized by wide-spread anatomical and physiological changes in an adult nervous system. During segmental regeneration of the annelid worm, *Lumbriculus variegatus*, neural morphallaxis involved a reorganization of sensory, interneuronal, and motor systems as posterior fragments gained a more anterior body position. A monoclonal antibody, Lan 3-2, which labels a neural glyco-domain in the leech, was reactive in *Lumbriculus*. In the worm, this antibody labeled neural structures, particularly axonal tracts and giant fiber pathways of the central nervous system. A 60 KDa protein, possessing a lumbriculid mannose-rich glycoepitope, was upregulated during neural morphallaxis, peaking in its expression at three weeks post-amputation. Peak upregulation of the Lan 3-2 epitope, or the protein possessing it, corresponded to the time of major neurobehavioral plasticity during regeneration.

Analyses of asexually reproducing animals also revealed induction of the Lan 3-2 epitope. In this developmental context, Lan 3-2 epitope upregulation was also confined

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* Reprinted from Mechanisms of Development 122(5): 721-732, Martinez VM, Menger GJ III, and Zoran MJ, Regeneration and asexual reproduction share common molecular changes: upregulation of a neural glycoepitope during morphallaxis in *Lumbriculus*.

to segments expressing both changes in positional identity and neurobehavioral plasticity, but these molecular and behavioral changes occurred prior to body fragmentation. These results suggest that the lumbriculid Lan 3-2 glycoepitope and proteins that bear them have been co-opted for neural morphallactic programs, induced both in anticipation of reproductive fragmentation and in compensation for injury-induced fragmentation.

Introduction

Since Trembley's studies on the regeneration of hydra, developmental biologists have sought to understand the cellular and molecular processes by which some animals readily reconstruct themselves following injury (Trembley, 1744; Sanchez-Alvarado and Newmark, 1998; Sanchez-Alvarado, 2000). In the early twentieth century, T. H. Morgan described two major forms of regeneration: epimorphosis and morphallaxis (Morgan, 1901). Epimorphosis is a pattern of regeneration that involves de-novo (via cell proliferation) generation of body parts or tissues. This form of regeneration can involve the dedifferentiation of adult structures and/or the activation of stem cell populations to form a mass of cells (blastema) that then differentiates. An example of epimorphosis is the regenerating amphibian limb (Goss, 1969). Morphallaxis, on the other hand, is a pattern of regeneration that involves the transformation of existing body parts or tissues into newly organized structures with little new cell growth, as in the reorganization of body parts in hydra following amputation (Holstein et al., 2003).

Much of the current understanding of developmental mechanisms underlying regeneration has emerged from studies using invertebrate model systems (Sanchez-

Alvarado, 2000). In fact, invertebrate animals are champions of regeneration, often rapidly regenerating much of their body from small isolated fragments or cell aggregates. This regenerative potential of invertebrates is especially evident within neural tissues. For example, while regeneration can occur within the central nervous system (CNS) of vertebrates, especially in amphibians (Lee, 1982; Clarke et al., 1988) and fishes (Mackler and Selzer, 1985), CNS regeneration is rapid and precise in most invertebrates. In contrast, following injury to their spinal cords, mammals are often permanently paralyzed (Puchala and Windle, 1977).

Striking regenerative powers, both epimorphic and morphallactic, are possessed by annelid worms, which reconstruct an adult animal from as little as three segments in a matter of weeks (Berrill, 1952). *Lumbriculus variegatus* has a remarkable capacity for regeneration of lost body segments (epimorphosis) and for neural plasticity within original body segments (morphallaxis) following injury-induced fragmentation. Lumbriculid body fragments regenerate eight new head segments and tails of variable lengths (Berrill, 1952; Drewes and Fourtner, 1990; Martinez et al., 2005b). Because of this differential segmental replacement between anterior and posterior buds, regenerating posterior body segments undergo an anatomical and physiological transformation to match their new, more anterior positional identity (Drewes and Fourtner, 1990; Myohara et al., 1999; Martinez et al., 2005b). Neurobehavioral transformations, or neural morphallaxis, are detected as changes in rapid escape behaviors and the neural correlates that underlie them. Rapid escape behaviors are mediated by three giant fiber pathways and their associated sensory inputs and motor outputs. Three segmentally-arranged giant axons, located in the

dorsal region of the ventral nerve cord, constitute through-conducting pathways (Günther and Walther, 1971) and are conserved among virtually all oligochaetes (Zoran and Drewes, 1987). The medial giant fiber (MGF) governs head-specific escape behavior (i.e., rapid anterior shortening), while paired lateral giant fibers (LGF) mediate rapid tail withdrawal (Drewes, 1999). Sensory inputs differentially activate the GF pathways along the anterior to posterior axis leading to the recruitment of head and tail specific behaviors (Drewes and Fourtner, 1989; Drewes, 1999). Neural morphallaxis in regenerating *Lumbriculus* fragments involves changes in touch sensory fields, giant axon diameter and conduction velocity, and motor system properties appropriate for the fragment's new body position.

A leech monoclonal antibody, Lan 3-2 (Zipser and McKay, 1981), was employed for analysis of neural epitopes upregulated during lumbriculid morphallaxis. In the leech, Lan 3-2 antibody reacts with mannose-rich glycoepitopes of multiple proteins. The Lan 3-2 glycoepitope has been implicated in critical neural developmental events such as synaptogenesis (Zipser et al., 1989; Song and Zipser, 1995; Huang et al., 1997; Tai and Zipser, 1998;1999; 2002). As demonstrated using antibody perturbation experiments, the Lan 3-2 mannosidic epitope mediates the defasciculation, sprouting, and arborization of sensory neuronal processes as they enter the CNS (Briggs et al., 1993; Zipser et al., 1994; Song and Zipser, 1995; Zipser, 1995). In addition, two neural cell adhesion proteins, LeechCAM and Tractin, possess the Lan 3-2 epitope and label neural pathways (Huang et al, 1997; Jie et al., 1999). Therefore, the developmental functions of a set of leech glycoproteins are, in part, dependent on the signaling properties conferred upon them by

their glycoepitopes, including Lan 3-2 (Johansen et al., 1985; Zipser, 1995;Tai and Zipser, 1999; Baker et al., 2003). In *Lumbriculus*, we have found that Lan 3-2 epitope-bearing proteins were expressed in neural tissues and were upregulated during neural morphallaxis. These changes in Lan 3-2 epitope expression were present prior to fragmentation during asexual reproduction and following fragmentation during injury-induced segmental regeneration.

Materials and Methods

Animals and Maintenance

Worms were purchased from Flinn Scientific, Inc. (Batavia, IL). They were housed in moderately aerated spring water, at a constant temperature of 16ºC ($\pm$1) in the dark. Worms were provided brown paper towel clippings for substrate and were fed spirulina powder and Tetramin staple flakes twice weekly.

To obtain experimental body fragments from specific body regions (anterior or posterior), worms were briefly anesthetized in 0.25 mM Nicotine in spring water. Segmental amputations were made at intersegmental boundaries with microdissecting scissors. Body fragments consisted of approximately 30 segments from the anterior third of the worm and 30 segments from the posterior third of the worm. Regenerating body fragments were maintained individually in containers of spring water at 16ºC. Although animals regularly fragment by asexual fission in laboratory cultures, the rate of fission is depressed in animals maintained with sufficient aeration and substrate at cool temperatures. To promote asexual fission, worms were exposed to an environmental

shift involving transfer to room temperature (22 ºC) and culture conditions that lacked paper substrate and aeration for 3-4 days. Worms were then returned to cultures at 16ºC.

Giant Fiber Sensory Field Mapping

Impulse conduction along giant nerve fibers was studied using non-invasive electrophysiological recordings (O'Gara et al., 1982; Drewes and Fourtner, 1990). Touch stimuli were delivered by a hand-held plastic probe. Medial giant fiber (MGF) and lateral giant fiber (LGF) action potential waveforms were distinguished based on previously reported spike characteristics (Drewes and Fourtner, 1989; 1990; Rogge and Drewes, 1993). Extracellular voltage recordings were obtained using a printed-circuit-board grid of electrodes and electrical signals were preamplified using a pair of differential recording amplifiers (100x gain, AC-coupled inputs). These spike recordings were digitized with a Powerlab A-D conversion system (ADInstruments, Inc.) and were analyzed on a G4 Macintosh computer (Apple, Inc.) using the Powerlab Chart software.

Spike conduction time between pairs of recording electrodes (5 mm pair spacing) was measured from peak-to-peak of giant fiber spike waveforms. Conduction velocity (m/s) was obtained by dividing the conduction distance by the spike conduction time. Individual means, based on 3-5 measurements per animal, were used in calculating group means. Non-invasive recording grids were also used to map giant fiber sensory fields. Segments of specific identity (e.g., segment number 50) were marked with a spot of water-insoluble ink from a fine tip pen (Sharpie). Individual segments were then touched with a probe and giant fiber responses were monitored electrophysiologically.

Immunocytochemistry

Worm Fragments were processed with monoclonal antibody Lan3-2, a mouse monoclonal antibody raised against the adult nervous system of the leech *Haemopis marmorata* (Zipser and McKay, 1981). Whole mount preparations were first pinned out on sylgard blocks while immersed in 0.25 mM nicotine in spring water, which blocks reflexive muscle movements and prevents autotomy (Lesiuk and Drewes, 1999). The VNC was exposed by removing the digestive tract, nephridia, and ventral blood vessel. Fragments were fixed in 4% paraformaldehyde, washed in PBS, dehydrated in ethanol, cleared in xylene, and rehydrated. The fragments were then incubated overnight at 4^{o}C with primary antibody (Lan 3-2, 1:10; 4G5, 1:10; Laz 6-56, 1:10; Laz 2-369, 1:10; provided by J.Jøhansen). Incubated fragments were washed extensively and incubated in either goat anti-mouse antibody conjugated to fluorescein (FITC, 1:1000; Sigma), rabbit anti-mouse antibody conjugated to Texas Red (1:1000; American Qualex), or horse anti-mouse antibody conjugated to peroxidase (HRP, 1:500; Vector Labs PI-2000). Double-labeled preparations were obtained by a subsequent incubation in a second primary antibody, anti-acetylated tubulin (1:1000; Sigma) and by using fluorescently conjugated subtype-specific secondary antibodies. A goat anti-mouse IgG FITC-conjugated secondary antibody was used for Lan 3-2 and a rabbit anti-mouse IgG_{2B} Texas red-conjugated secondary antibody (American Qualex) was used for the acetylated tubulin antibody. Preparations were imaged using an Olympus inverted microscope, DIC optics, and a Hamamatsu CCD camera. Images were captured with Simple PCI software (C-Imaging, Inc.) and imported into Adobe Photoshop 6.0.

Giant Fiber Injections

Whole mount preparations were pinned out on sylgard blocks while immersed in 0.25 mM Nicotine (in worm saline) and treated as described above. Individual giant axons were injected via micropipettes containing a 3% lucifer yellow/fast green solution using a picospritzer (General Valve). Following a 30 min diffusion period, preparations were fixed and processed for imaging.

SDS-Page and Western Blotting

Experimental fragments were cultured for 1-9 weeks post-amputation. Prior to homogenization, newly formed head and tail pieces where excised and discarded to remove factors specific to epimorphic tissues. Worm fragments (approximately 25 pieces; 300 mg wet weight) were then homogenized in osmotic lysis buffer (10 mM Tris, pH 7.4 and 0.3% SDS) supplemented with a cocktail of protease inhibitors (20 mM AEBSF, 1 mg/ml leupeptin, 0.36 mg/ml E-64, 500 mM EDTA, 5.6 mg/ml benzamide) and nucleases (50 µg/ml RNase, 100 µg/ml DNase in 5mM $MgCl_2$ and 10mM Tris-Cl, pH 7.0). All steps of protein sample preparation were completed on ice. All experiments of regenerating worms were replicated across at least three separate populations of animals. SDS-Page was performed according to standard procedures (Laemmli, 1970). Electroblot transfer was performed as in Towbin et al. (1979) using the Hoeffer system (Amersham) and electroblotting to 0.2 µm Nitrocellulose (Bio-Rad). Blots were incubated with diluted antibody (Lan 3-2, 1:10, J.Jøhansen; Anti-α-Tubulin, 1:1000, Sigma) and visualized using anti-mouse alkaline phosphatase conjugated secondary antibody (1:300; Vector). In some experiments, immunoblots were probed with a

primary antibody that had been preincubated with methyl α-D-mannopyranoside (0.5 M). The signal was then developed with BCIP/NBT (tablets; Sigma). Stained gels or blots were digitized using a Nikon image capturing system and were analyzed using NIH Image densitometry analysis.

Statistics

Two-tailed student's t-tests (Microsoft Excel) or ANOVA (Statistica, Inc.) were used for statistical analysis. Data are presented as mean plus or minus standard deviation (s.d.) or standard error of the mean (s.e.m.) as indicated. Statistical significance was $p<0.05$.

Results

Neural Morphallaxis in Lumbriculus variegatus

Body fragments of 30 ± 1 (mean ± sem) segments were amputated from the anterior third and the posterior third of adult worms of approximately 150 segments (~ 5 cm in length; Fig.10a). Segmental regeneration was asymmetric such that regenerating fragments, from both anterior and posterior body regions, consistently regenerated 7-8 new head segments. However, the number of new tail segments differed between populations, with anterior fragments producing 59 ± 5 tail segments and posterior fragments producing 27 ± 5 tail segments (Fig. 10a).

Escape behaviors in *Lumbriculus* are mediated by mechanisms that vary along the anterior-posterior axis (Drewes and Fourtner, 1989; Drewes, 1999). Although giant fiber pathways allow impulse conduction along the entire length of the animal (Drewes, 1984; Zoran and Drewes, 1987), the MGF is excited by touch sensory stimuli only to

anterior segments. Tactile stimulation of the anterior 1/3 region of an intact animal's body wall (44 ± 2.1segments, ~29% of total segments) triggered only MGF spikes, resulting in head withdrawal (Fig.10a). In contrast, the two LGFs were activated by touch stimuli only to the posterior 2/3 region of the worm (93.2 ± 2.5 segments, ~62% of the total segments) resulting in tail withdrawal behavior. MGF and LGF sensory fields coexisted in an overlap region of 12.8 ± 2.3 segments, the equivalent of 9% of body segments (Fig.10). Thus, MGFs are interneurons that mediate sensory inputs for escape in the anterior third of animal; whereas, LGFs integrate these inputs in the posterior two-thirds.

At the time of amputation, fragments of 30 segments removed from anterior and posterior regions possessed exclusively MGF and LGF sensory fields, respectively (Fig.10a). During the subsequent 3 weeks of neural morphallaxis, fragments exhibited transformations in their escape reflex circuitry (i.e., sensory fields). In anterior fragments, LGF sensory fields emerged in approximately 1/3 of the posterior-most segments, while the MGF field was lost in only one or two original segments (Fig. 10a). Neural morphallaxis of sensory fields was more dramatic in posterior fragments, where MGF fields developed in 2/3 of the anterior-most segments of the original fragment and LGF field receded from 1/3 of these segments. In both fragment groups, a region of sensory field overlap was present following morphallaxis, where none existed previously (Fig. 10a).

Giant fiber conduction velocities, in both anterior and posterior fragments, were significantly altered following amputation. Significant differences in LGF conduction

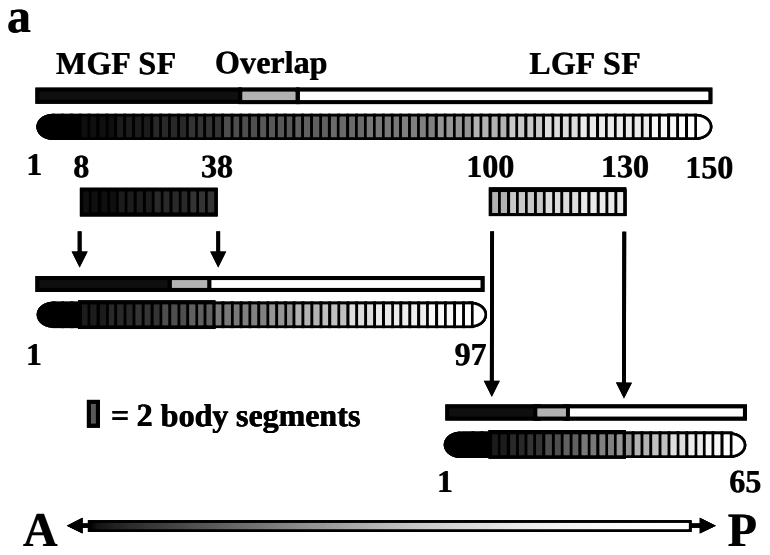

Figure 10 – Neural morphallaxis during regeneration following injury-induced fragmentation.

a. Segmental Regeneration in Lumbriculus. Whole animals of approximately 150 segments in length were cut into pieces. Two populations of fragments, one from the anterior (~segment 8-38) and one from the posterior (~segment 100-130) regions of the worm, were maintained. All other pieces were discarded. Amputated fragments always regenerated 7-8 segments of head and variable numbers of tail segments (~30-60). Posterior fragments became more anteriorly positioned following segmental regeneration. Numbers represent segmental identity and shading implies differences in anterior-posterior position. One box = 2 body segments. Anterior = A; Posterior = P.

Sensory field maps (rectangular boxes above worm illustrations) were determined. Prior to amputation, stimulation of the anterior 1/3 region (segment 1-43) activated only a medial giant fiber (MGF) spike resulting in a head withdrawl. Stimulation of the posterior 2/3 region (segment 58-150) activated only lateral giant fiber (LGF) spiking causing a tail withdrawal. An area of both both MGF and LGF spike activation (sensory field overlap; indicated by grey region of sensory field map) was detected, on average, between segments 44-57 (n=6). Following 3 weeks of regeneration, medial giant fiber spikes were activated by touch to the original segments of posterior fragments (darker outlined boxes), thus gaining sensory fields previously absent. Although not pervasive, fragments removed from the anterior 1/3 region also gained lateral giant fiber sensory fields that were absent prior to amputation.

velocities in both anterior and posterior fragments were detected following 3 weeks of morphallaxis (Fig. 10b). In posterior fragments, LGF conduction velocities decreased 26.7% as compared to intact control animals. Previous studies of neural morphallaxis have shown that MGF and LGF axonal diameters are significantly altered concomitant with changes in spike conduction velocity (Drewes and Fourtner, 1990; Martinez et al., 2005b). Therefore, neural morphallaxis was characterized by changes in giant fiber

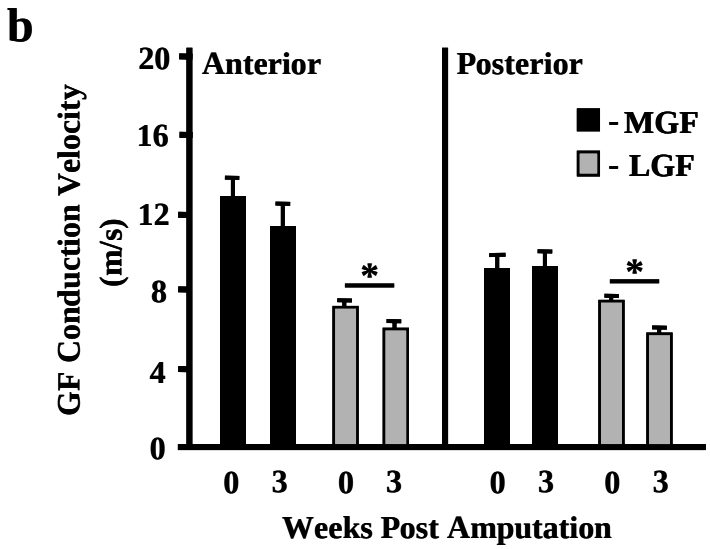

Figure 10 – (Continued).

b. Giant fiber conduction velocity in anterior and posterior fragments during segmental regeneration. Prior to amputation in anterior fragments, MGF conduction velocities (solid bars) measured 12.9 ± 0.7 m/s and LGF conduction velocities (gray bars) measured 7.2 ± 0.1 m/s in the anterior region of the worm (n=8; p<0.0001). Three weeks post-amputation, MGF and LGF conduction velocities returned to normal rates (p<0.01). In posterior fragments prior to amputation, MGF conduction velocities measured 9.1 ± 0.1 m/s and LGF conduction velocities measured 7.6 ± 0.4 m/s in the posterior region (n=8; p>0.05). Following amputation, LGF velocities decreased (p<0.0001). Bars equal group mean ± s.d.

diameter, conduction velocity, and sensory field maps that emerged several days post-amputation and approached normal levels (i.e., those appropriate for segmental position) three weeks following fragmentation (Drewes and Fourtner, 1990; Martinez et al., 2005b).

Lan 3-2 Antibody Recognizes an Epitope Expressed in Neural Tissues

We performed an immunohistochemical screen using four leech antibodies (4G5, Laz 6-56, Laz 2-369, and Lan 3-2) to test for cross-reactivity within the *Lumbriculus* nervous system. These individual antibodies label various galactosidic and mannosidic epitopes born on leech glycoproteins (Zipser and McKay, 1981; McKay et al., 1983; Bajt et al., 1990; Johansen et al., 1992). Since these glycoproteins are present on the

surface of many sensory axons in leech and are thought to function in target selection processes, we used them in an attempt to identify proteins correlated with lumbriculid morphallaxis. Since neural morphallaxis involved changes in giant fiber form and function, we screened specifically for staining of giant interneuronal pathways. The Lan 3-2 antibody proved to be most reactive within the lumbriculid nervous system. Lan 3-2 immunostaining was localized to periaxonal regions of the giant fiber pathways; that is, diffuse staining was detected near the extracellular surface of the giant axons and their surrounding glial sheaths (Fig. 11a-c). Lan 3-2 staining was localized primarily to lateral giant axons within the ventral nerve cord and distinct axons within the segmental nerves (Fig. 11d). Confocal microscopy revealed particularly intense staining adjacent to the LGFs (Fig. 12a). Staining outside the CNS included processes in segmental nerves, flattened circular staining patterns in the muscle (reminiscent of synaptic boutons), and small cell bodies and processes in the body wall indicative of sensory neurons (Fig. 2d-g). However, the number of sensory neurons stained indicated that Lan 3-2 recognizes only a subset of these peripheral neurons. Dual labeling of *Lumbriculus* central nervous system with monoclonal antibodies to both acetylated tubulin and Lan 3-2 confirmed that Lan 3-2 labeled only a specific subset of neuronal tracts (Fig. 11e). Lan 3-2 stained preparations also revealed fine processes within the neuropile of the ventral nerve cord (Fig. 11h). Using confocal microscopy, a regular pattern of Lan 3-2 staining along the length of the giant fibers was detected. This pattern included an oval region of reduced Lan 3-2 reactivity surrounding a cluster of intensely stained puncta, a pattern repeated with a regular spacing of approximately 40-50 μm or four times per segment.

Intracellular lucifer yellow injections demonstrated that axonal collaterals, fine dendrite-like processes on the ventral surface of annelid giant axons and sites of sensory/motor

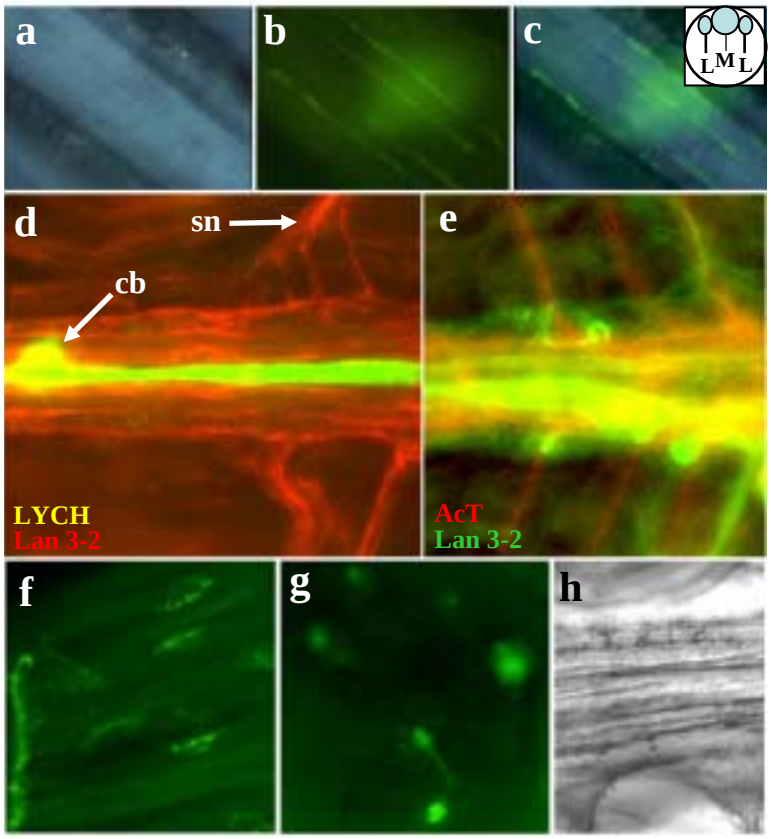

Figure 11 – Lan 3-2 epitope is expressed on neural structures.

a-c. Lan 3-2 expression within the Lumbriculus nervous system using fluorescence immuno-labeling of whole mount preparations. Lan 3-2 was localized to the periphery of giant fiber pathways, particularly the LGF. Inset in c represents a cartoon depicting the presence of three giant fibers, one MGF (M) and two LGFs (L), beneath the dorsal surface of the nerve cord. Dorsal surface of the nerve cord is facing toward the reader in all whole-mount images (Nomarski, a; Lan 3-2 FITC, b; Merged, c). Scale bars = 15 µm.

d-e. Lan 3-2 epitopes were localized to axonal tracts within the ventral nerve cord and segmental nerves (sn). The MGF in the preparation in panel d was filled with Lucifer yellow (LYCH) to demonstrate giant fiber orientation. The MGF cell body (cb) is indicated (different focal plane). Dual immuno-labeling (Lan 3-2-FITC/acetylated tubulin (AcT)-Texas Red) of a whole mount preparation in panel e revealed Lan 3-2 epitopes associated with specific segmental nerve tracts. Note that some tracts were positive for both Lan 3-2 and AcT, while others showed only AcT staining. Scale bars = 20 µm.

f-h. Lan 3-2 epitopes were expressed on synaptic bouton-like structures within the body wall musculature (f; FITC-conjugated 2^o), on cell bodies, perhaps sensory neurons, within the body wall (g; FITC-conjugated 2^o), and on small branched processes (arrows) that extended within the nerve cord neuropile (h, HRP-conjugated 2^o). Scale bars = 10 µm.

synaptic contacts, were regularly spaced along the length of the giant fibers (Fig. 12b). These axonal collaterals were also arranged four per segment with intercollateral spacing of approximately 40-50 µm.

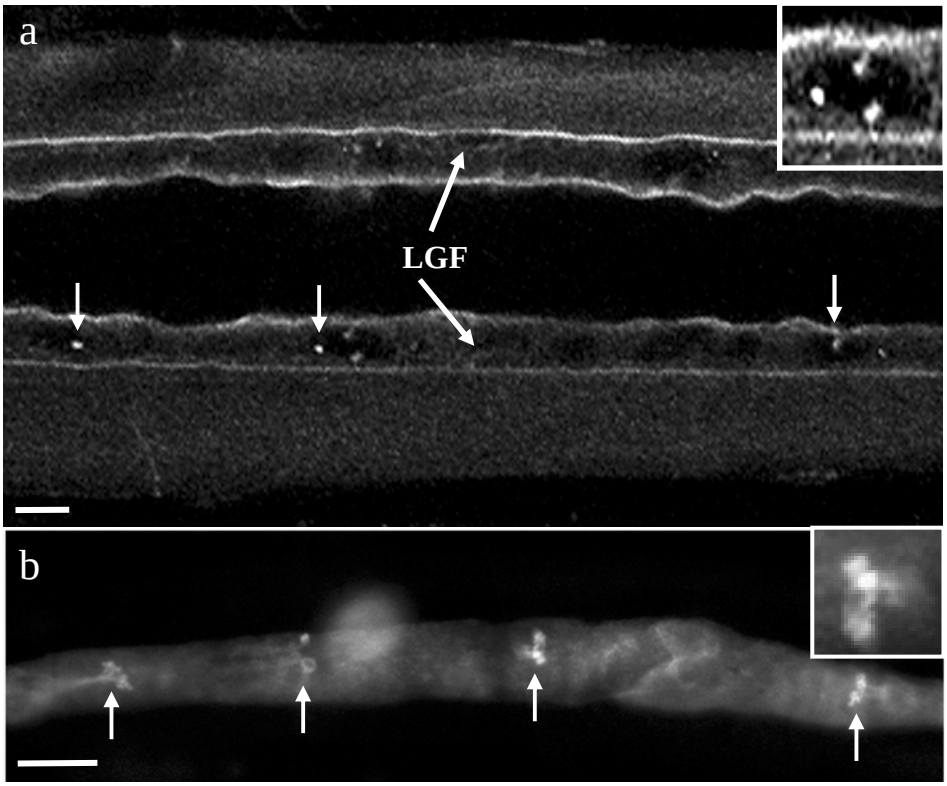

Figure 12 – Lan 3-2 epitope is expressed at giant interneuronal collaterals.

a. Confocal imaging of ventral nerve cord revealed uniform staining of the two lateral giant fibers (LGFs) with the Lan 3-2 antibody. Regularly spaced punctate staining patterns were evident along the length of the interneuronal axons (arrows). These clusters of intensely stained puncta were surrounded by a region approximately 10 µm in diameter that was devoid of Lan 3-2 immunoreactivity (insert). Scale bar = 10 µm.

b. Lucifer yellow-filling of a giant interneuron also revealed regularly spaced collateral extensions along its ventral surface (arrows). The spacing of these collateral extensions was similar to the punctate Lan 3-2 expression in panel a. Collateral branches appeared as short extensions from the basal surface of the axon that branched into multiple lobes (inset). Scale bar = 10 µm.

Immunocytochemical staining of whole mount preparations revealed an anterior-posterior gradient in the intensity of Lan 3-2 antibody immunofluorscence in intact, control worms (Fig. 13a-d). Although small, these differences in the fluorescence

intensity of the ventral nerve cord between anterior and posterior segments were statistically significant (Fig. 13e). Immunocytochemistry was also performed on lumbriculid body fragments over the time course of segmental regeneration and neural morphallaxis. However, this fluorescence staining approach was not sensitive enough to detect changes in levels of Lan 3-2 epitope expression in the ventral nerve cord regions during fragment regeneration. Therefore, Lan 3-2 epitope induction during neural morphallaxis was investigated using western blot analysis.

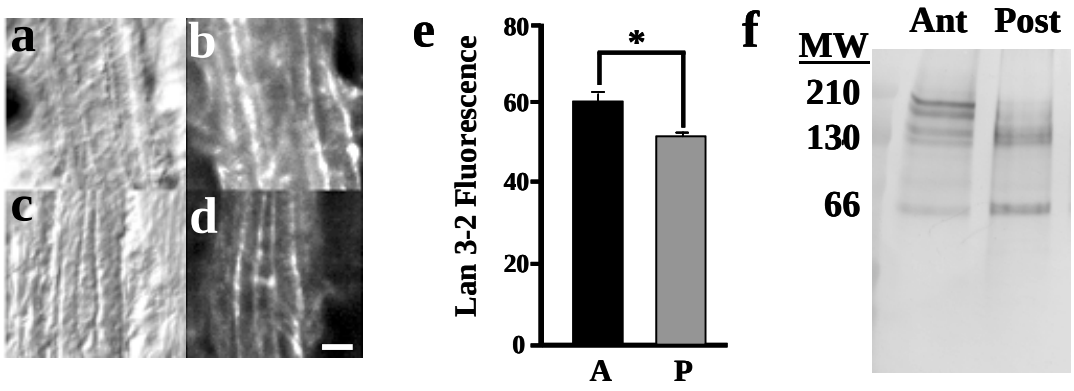

Figure 13 – Anterior-posterior differences in Lan 3-2 expression.

a-d. Immunocytochemical analyses of Lan 3-2 positive proteins in intact (control) animals revealed a difference in expression between anterior (Nomarski - a; FITC - b) and posterior segments (Nomarski - c; FITC - d). Anterior segments had a greater intensity of Lan 3-2 staining than segments found in the posterior region. Scale bar = 15µm.

e. Differences in ventral nerve cord (VNC) fluorescence intensity in both anterior and posterior regions were quantified. Posterior segments exhibited a significantly lower level of VNC Lan 3-2 fluorescence ($p<0.0001$).

f. Immunoblot analysis of protein extracts from anterior and posterior body regions. Lan 3-2 positive proteins were expressed differentially between anterior and posterior regions. Pairs of protein bands at approximately 210 and 130 KDa were detected in anterior segment extracts. In contrast, the 130 KDa doublet of protein bands and a protein band of approximately 60 KDa were detected in posterior segment extracts at levels greater than anterior extracts.

Multiple Proteins in Lumbriculus *Possess the Lan 3-2 Epitope*

Lumbriculid Lan 3-2 glycoepitopes were born on several proteins of varying molecular weight in intact (control) worms detected by immunoblot analysis (Fig. 13f).

Although most, if not all, of these proteins were present along the length of the animals body, significant differences in the abundance of Lan 3-2 positive-proteins were observed among anterior and posterior body positions. A doublet of proteins of approximately 210 KDa were abundant in anterior body segments. Another pair of protein doublets of approximately 130 and 60 KDa was more abundant in posterior segments. These characteristic Lan 3-2 expression profiles indicated the existence of anterior-posterior positional gradients in the expression of Lan 3-2 positive proteins. This result, that multiple proteins in adult worms share the Lan 3-2 glycoepitope, may account for the lack of detectable changes in Lan 3-2 immunofluoresence, especially if only a subset of these proteins were altered during neural morphallaxis.

Lan 3-2 is a Glycoepitope That Is Transiently Upregulated During Morphallaxis

The 60 KDa, Lan 3-2 positive glycoprotein was markedly upregulated at 3 and 5 weeks post-amputation in posterior fragments. The expression of this Lan 3-2 positive protein then diminished to relatively undetectable levels by week seven of regeneration (Fig. 14a-b). Although some upregulation of the 60 KDa glycoprotein was also observed in anterior fragments, it was only a fraction of the induction seen in posterior fragments. Thus, the induction of these epitopes coincided with expression of anatomical and physiological changes within the nervous system. We cannot currently explain the overall decline in epitope detection with the Lan3-2 antibody at week 9 post-fragmentation. Future studies varying fragment culture conditions will address this issue.

Previous studies in leech characterized Lan 3-2 positive antigens as mannose-rich glycoproteins and revealed that antibody binding involves a mannose epitope within the carbohydrate domain (Flanagan et al., 1986; Flaster et al., 1983; McGlade-McCulloh et al., 1990; McKay et al., 1983). To determine whether the Lan 3-2 antibody also binds to

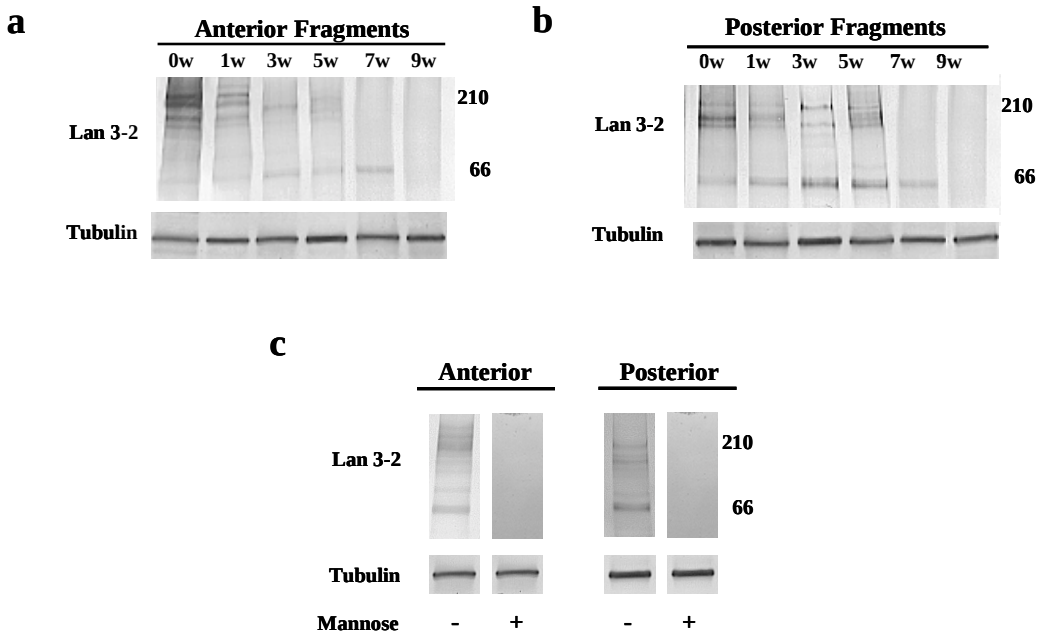

Figure 14 – The Lan 3-2 antibody binds to glycoproteins that are differentially upregulated following fragmentation.

a-b. Immunoblot analysis demonstrated the presence of Lan 3-2 epitopes in both anterior and posterior fragments. Protein extracts from anterior (a) and posterior (b) fragments were generated at different time points during 9 weeks of regeneration. Proteins of 60KDa possessing the Lan 3-2 epitope were enriched in posterior fragments, and to a lesser extent in anterior fragments. The 60KDa protein was transiently upregulated with peak expression at 3 weeks following amputation. Tubulin antibody was utilized as a loading control.

c. Protein extracted from three week old regenerating fragments were immunobloted with Lan 3-2 antibody either in the presence of 0.5 mM mannose (+) or without mannose (-). Lan 3-2 positive epitopes, present in blots from both anterior and posterior control fragments (- mannose), were no longer visible in immunoblots following treatment (+ mannose), suggesting that the lumbriculid Lan 3-2 epitope is a glycodomain. Similar experiments using a tubulin antibody demonstrated that the binding of this antibody was not competed on mannose-treated immunoblots.

a mannose epitope of lumbriculid proteins, immunoblots were probed with antibody that had been preincubated with methyl α-D-mannopyranoside (0.5 M). Staining of

lumbriculid proteins with the Lan 3-2 antibody was attenuated following mannoside treatment (Fig. 14c), suggesting that the Lan 3-2 antibody recognizes a mannose-rich glycoepitope.

Lan 3-2 Positive-Proteins Are Upregulated During Asexual Reproduction

To confirm that upregulation of the Lan 3-2 positive glycoprotein was a fundamental component of molecular changes in neural morphallaxis, and not simply a result of a general injury response, we observed its expression during another developmental event involving neural morphallaxis and changes in segmental identity - asexual reproduction. *Lumbriculus* reproduces primarily through a developmentally-regulated fragmentation process called architomy (Berrill, 1952; Martinez et al., 2005b).

Using an environmental shift to induce asexual reproduction, a cumulative total of 88% of worms across three populations fragmented by the third week after onset of the induction protocol (Fig. 15a). Using this experimental paradigm, we found that two body fragments were consistently produced during architomy at a fission plane formed at segment 48 ± 10.2 (in worms with 150 segments; Fig. 15b). This predictable fission (architomy) site at segment 48 was located virtually at the center of the zone of MGF/LGF sensory field overlap (Fig. 10a). We have determined that neural morphallactic changes occur in anticipation of fragmentation during asexual reproduction in *Lumbriculus* (Martinez et al., 2005b). Here, using sensory field mapping, we detected a significant expansion of the MGF sensory field and retraction of the LGF sensory field posterior to the architomy site (Fig. 15b). As the fission plane

developed, the zone of sensory field overlap expanded from 12.8 ± 2.3 segments (mean ± s.d.) to 29.5 ± 8.9 segments. Furthermore, an area of exclusively MGF sensory field

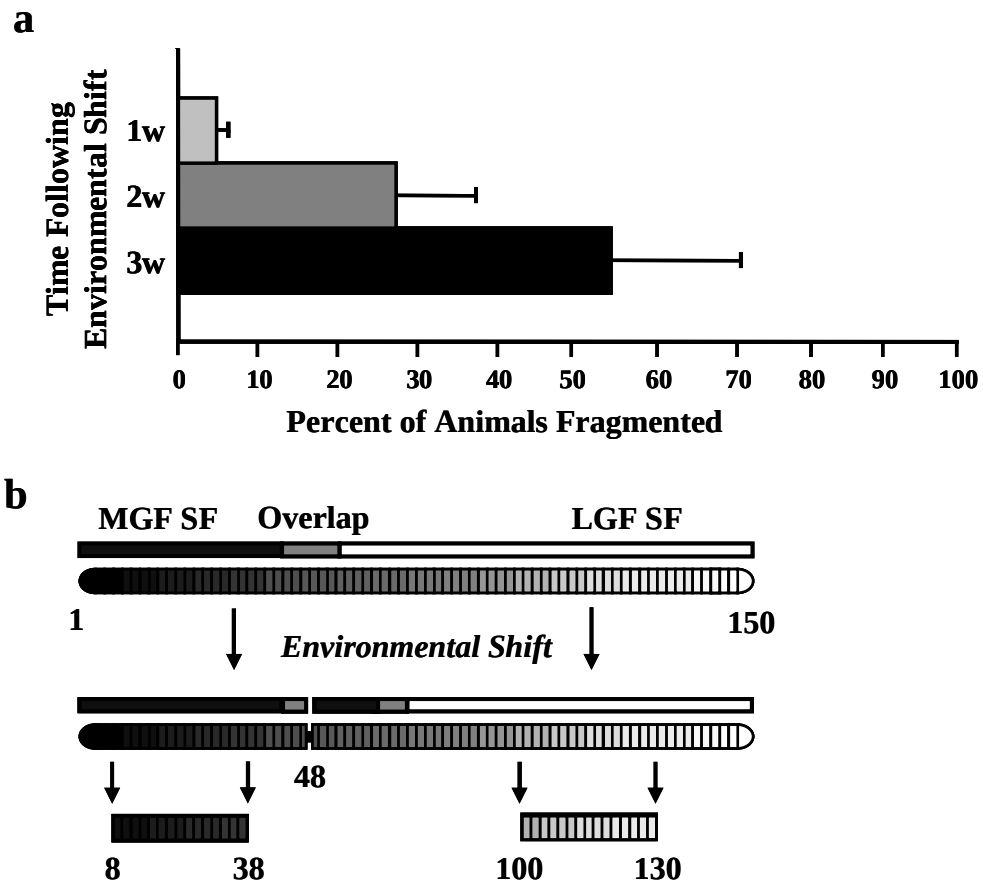

Figure 15 – Lan 3-2 epitope upregulation precedes fragmentation (architomy) during asexual reproduction.

a. Asexual reproduction was induced in whole animals by an environmental shift in culture conditions (see Experimental Procedures). Following the environmental shift, over 88% of the animals fragmented by week 3 (n = 3 experimental populations).

b. During asexual reproduction, fission zones were formed two weeks following the environmental shift. These architomy sites were consistently produced at segment 48 ± 10 in animals of approximately 150 segments ($p<0.0001$; n = 37). Giant fiber sensory fields expanded prior to fragmentation during asexual reproduction and the zone of MGF/LGF sensory field overlap was disrupted by newly emerging sensory inputs. Sensory field overlap expanded from 12 segments in controls to 29 segments in animals exposed to the environmental shift protocol. In 10-15 segments posterior to the architomy site, only MGF spikes could be evoked by tactile stimulation (n = 6). Segmental sites of tissue collection for protein extraction and immunoblots were in the anterior 1/3 region (~ segment 8 – 38) and the posterior 2/3 region (~ segment 100 – 130). MGF/LGF sensory fields are mapped above worm images in rectangular boxes. Anterior-posterior direction is indicated by color gradient. One box = 2 body segments.

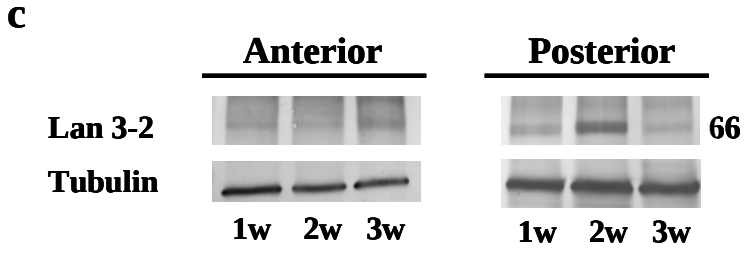

Figure 15 – (Continued).

c. Protein extracts from the anterior 1/3 (~ 30 segments) and the posterior 2/3 region (~ 30 segments) of asexually reproducing animals were collected at 1 week (1w), 2 weeks (2w), and 3 weeks (3w) following environmental shift. The Lan 3-2 epitope of 60KDa was detected at low levels in all extracts. However, this protein was highly upregulated in posterior extracts two weeks after induction of asexual reproduction (2w).

(10-15) segments just adjacent and posterior to the fission plane was present in animals with late-stage fission plane formation, many days prior to fragmentation. During asexual reproduction, changes in sensory field were accompanied by a significant upregulation of the Lan 3-2 glycoepitope. Two weeks prior to fragmentation the 60 KDa protein was robustly induced in segments posterior, but not anterior, to the architomy site (Fig. 15c). Therefore, Lan 3-2 glycoepitope upregulation was specific to segments involved in neural morphallaxis and was not a result of body injury or wound healing.

Discussion

Neural morphallaxis in *Lumbriculus variegatus*, associated with body fragmentation, involves a transformation of the adult nervous system as it acquires a new anterior-posterior positional identity and concomitant neurobehavioral constraints. Body fragments of *Lumbriculus* regenerate a new head of only eight segments and a new tail of variable length, thus original segments from posterior fragments often acquire a more anterior body position. One aspect of this change in segmental identity involves the

morphallactic plasticity of sensory, interneuronal, and motor pathways that mediate anterior- and posterior-specific behaviors (Drewes and Fourtner, 1990; Martinez et al., 2005b). Morphallaxis is characterized by reorganizational plasticity, without new neurogenesis (Morgan, 1901; Drewes and Fourtner, 1990; Myohara et al., 1999; Yoshida-Noro et al., 2000). Neural morphallaxis, during segmental regeneration (epimorphosis), is correlated with changes in sensory fields for the activation of escape reflexes, alterations in giant axon size, and synaptic plasticity at electrical and chemical synapses within the escape neural circuit (Drewes and Fourtner, 1990; Martinez et al., 2005b). Additionally, neural morphallaxis occurs during the induction of asexual reproduction. Although cellular correlates of neural morphallaxis have been described, little is known about the molecular changes that mediate this developmental event. Here, we have identified molecular markers of neural morphallaxis, proteins possessing the lumbriculid Lan 3-2 glycoepitope, and demonstrated that their induction is temporally correlated with neurobehavioral plasticity.

The Lan 3-2 monoclonal antibody was developed in leech and labels a glycoepitope present on neural cell adhesion molecules found on sensory neurons during development (Huang et al., 1997). In *Lumbriculus*, the temporal expression of the Lan 3-2 positive glycoproteins suggests that these proteins or post-translational modifications (i.e., glycosylation events) of these proteins are induced during neural morphallaxis. Western blot analysis of regenerating fragments indicated the differential upregulation of 60KDa proteins for many weeks post-amputation, a time corresponding to changes in the worm's neural anatomy and physiology (Fig. 16a). These cellular and molecular

changes occur in parallel with new head and tail bud formation. However, newly formed segmental tissue was amputated just prior to protein extraction and do not account for changes in protein levels described here. Thus, the Lan 3-2 epitope was present in tissues experiencing morphallaxis. The possible induction of the Lan 3-2 epitope in epimorphic tissue (buds) remains to be determined. These data are interesting in the light of previous results from the leech system which demonstrated that neural cell adhesion molecules are differentially glycosylated during development with the Lan 3-2 epitope (Huang et al., 1997; Johansen and Johansen, 1997; Jie et al., 1999; 2000) and perturbation of this epitope results in a disruption of sensory afferent defasciculation and abnormal formation of synaptic contacts (Zipser et al., 1989; Zipser et al., 1994; Song and Zipser, 1995; Baker et al., 2003). As with neurobehavioral plasticity, the lumbriculid Lan 3-2 glycoepitope was also elevated during asexual reproduction (Fig. 16b). Interestingly, Lan 3-2 epitope expression in this reproductive context anticipated fragmentation (architomy) events and occurred in segments undergoing an expansion of MGF sensory field and a retraction in LGF sensory field. The fact that neurobehavioral plasticity and changes in neural glycoprotein expression exhibited precise temporal and spatial correlation, in two distinct life history contexts, suggests that the Lan 3-2 glycoepitope is part of a developmentally-regulated cascade that plays a direct or indirect role in neural morphallaxis.

Since neural morphallaxis was present during both injury-induced segmental regeneration and environmentally-induced asexual reproduction, a common developmental mechanism has been co-opted to serve both of these processes. Several

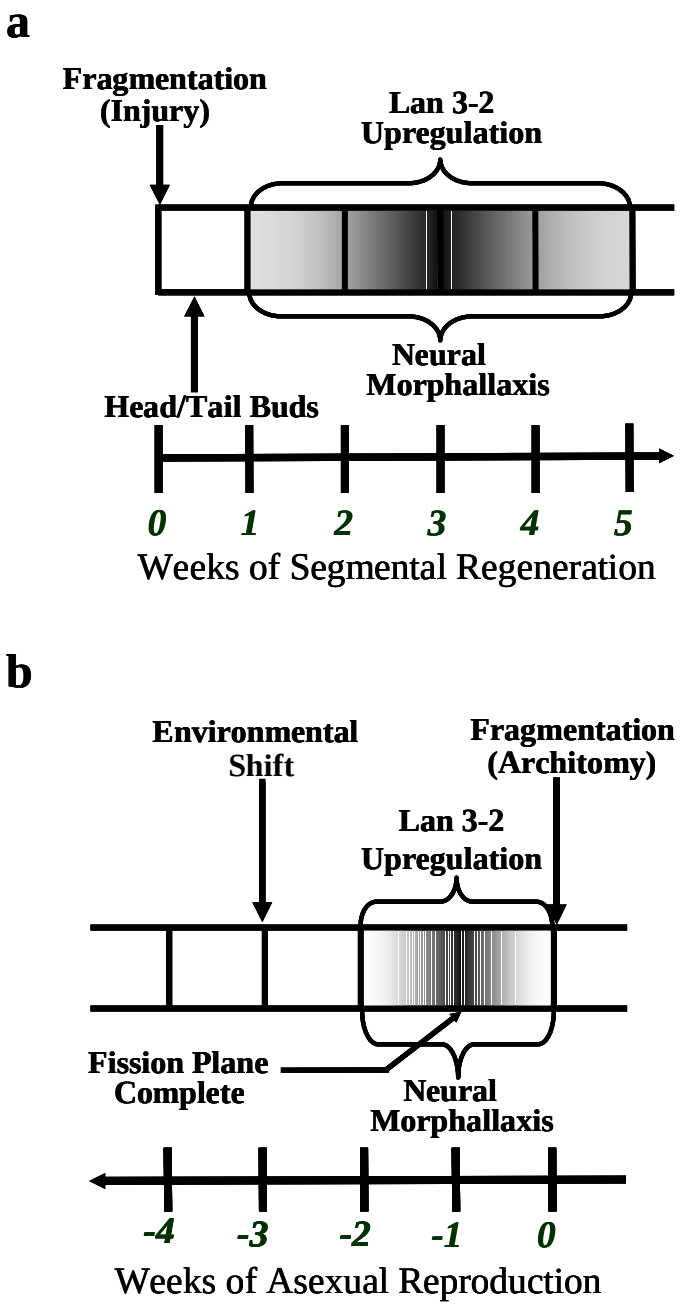

Figure 16 – Lan 3-2 epitope is upregulated during anticipatory and compensatory neural morphallaxis.

Neural morphallaxis (changes in neuroanatomy, physiology and behavior) was observed during both injury-induced regeneration (a) and asexual reproduction (b). Neural morphallactic changes associated with asexual reproduction appeared 1-2 weeks prior to architomic fission. During segmental regeneration, in contrast, neural morphallactic changes were not detectable until 1-2 weeks after amputation (injury). Thus, morphallaxis of the nervous system was produced by anticipatory regenerative mechanisms during asexual reproduction; whereas, morphallaxis was retroactively-induced during segmental regeneration following injury to compensate for lost body parts and changing segmental position. Lan 3-2 epitope expression (shaded area of boxes) was differentially upregulated prior to architomy and following injury. The induction of this neural glycoepitope correlated with both anticipatory and compensatory neural morphallaxis.

studies of regeneration in other annelid worms have indicated that the nervous system plays an important role in triggering some regenerative events (Drewes and Fourtner, 1991; Bely, 1999; Myohara et al., 1999; Yoshida-Noro et al., 2000). In *Lumbriculus*, the sites of asexual fission (architomy) occur consistently within the zone of giant fiber sensory field overlap (approximately segment 50) in worms of 150 segments. Morphallactic transformations of giant fiber anatomy and function emerge prior to observable fission plane formation in segments destined to change their positional identity. These observations again suggest a fundamental link between gradients of neural behavioral mechanisms and the processes that regulate reproductive and regenerative programs.

Immunohistochemical analysis revealed expression of the lumbriculid Lan 3-2 epitope on neural structures. In *Lumbriculus*, the Lan3-2 epitope was localized to distinct axonal processes within the ventral nerve cord, segmental nerves, and body wall musculature. Lan 3-2 immunocytochemical localization within the nerve cord was associated with the lateral giant fibers or their ensheathing neural glial cells, neural structures that undergo dramatic changes during neural morphallaxis. Importantly, the Lan 3-2 epitope was enriched on giant fiber axonal collaterals, which are the synaptic sites of sensory input and motor output in oligochaete giant axons (Günther and Walther, 1971; Zoran and Drewes, 1987) and likely the sites of cellular plasticity correlated with changes in sensory fields. Neural morphallaxis in posterior segments involves a rapid switch from exclusively LGF to a largely MGF sensory field in less than one week, indicating the involvement of extensive synaptic plasticity. In invertebrates, widespread

changes in synaptic connectivity are especially evident during regenerative events following injury (Bulloch et al., 1984; Cohan et al., 1987; Ambron and Walters, 1996; Chang and Keshishian, 1996; Szabo et al., 2004). Since the Lan 3-2 epitope has previously been implicated in synaptogenic events (Zipser et al., 1994; Zipser, 1995) and is expressed at putative sites of synaptic contact in *Lumbriculus*, it will be interesting to determine whether or not Lan 3-2 positive glycoproteins are directly involved in this novel form of neural plasticity.

In segmented animals, including annelids, anterior-posterior gradients of body form and function are established early in embryonic development. Many cellular and molecular mediators of these gradients have been established (Nusslein-Volhard et al., 1987; Shankland, 1991; 1994; 2000; Patel et al., 1989; Patel, 1994). Still, little is understood about adult cellular and molecular programs governing regeneration and changes in positional identities along the anterior-posterior axis during events such as morphallaxis. Several insights into these mechanisms emerged from our studies. First, developmental programs recruited during asexual reproduction may benefit from their anticipatory nature with precisely orchestrated and efficient signaling events. Neural morphallaxis prior to architomic fission took place over approximately one week, with a tight spike of Lan 3-2 epitope or Lan 3-2 positive protein upregulation and a rapid event of behavioral plasticity. On the contrary, developmental cascades required for repair and regeneration of damaged tissue are likely compromised in their speed and efficiency of expression due to the compensatory nature of their recruitment. Neural morphallaxis induced as a result of injury occurred over a period greater than 4-5 weeks, with Lan 3-2

epitope upregulated over this entire period and a relatively gradual neurobehavioral transformation (Fig. 16a).

A long-standing question in developmental biology has been centered on the evolutionary origins, and potential phylogenic relationships, between asexual reproduction and regeneration (Bely, 1999; Bely and Wray, 2001; Sanchez-Alvarado, 2000). Our results demonstrate that cellular mechanisms of morphallaxis have been co-opted for both of these developmental events in *Lumbriculus*, where reorganization of its metameric nervous system accompanies changes in positional identity. Shared temporal and spatial patterns of Lan 3-2 epitope expression during both life history events indicate that common molecular mechanisms may underlie them. Future identification of the Lan 3-2 positive-proteins and the molecular events they mediate will add new insight into the evolutionary origins of these events and determine whether or not these programs were co-opted for reproduction from more ancient regenerative processes, or vice versa.

CHAPTER IV

EFFECTS OF NERVE INJURY AND SEGMENTAL REGENERATION ON THE CELLULAR AND MOLECULAR CORRELATES OF NEURAL MORPHALLAXIS

Overview

Axonal injury results in a cascade of signaling events which determine the success or failure of functional regeneration of a synapse. Using *Lumbriculus variegatus*, a highly successful regenerating model system, we demonstrated the influence of axonal injury on neural morphallaxis, a type of regeneration which involves reorganization of synaptic connections that are appropriate for changes in the animal's anterior-posterior position. Previously we described cellular and molecular events which are characteristic of neural morphallaxis. Using experiments in which we have generated animals with segmentally regenerating ectopic heads and experiments in which we've blocked regeneration or reproduction with boric acid, we have demonstrated that damage to the nerve cord is necessary and sufficient for induction of neural morphallaxis. However, although nerve damage is necessary for induction, it is not sufficient for maintenance of morphallactic events. Thus, we present an example of synaptic plasticity that is highly influenced by cellular and molecular signals which arise as a result of the cells response to injury or stress.

Introduction

Recovery of neural function following nervous system damage depends on a sequence of cellular and molecular events triggered by the initial injury (Ambron and Walters, 1996; Brecknell and Fawcett, 1996; Moffett, 1996; Kumar et al., 2001; Fenrich and Gordon, 2004; Dinsmore and Mescher, 1998). These cellular events include the activation of growth factor-mediated signal transduction pathways (Brecknell and Fawcett, 1996) and upregulation of inducible transcription factors (Raivich et al., 2004). The cellular responses generated by neural injury include growth, synaptic modulation, and long-lasting compensatory changes in excitability (Ambron and Walters, 1996; Kandel, 2001). These cellular and molecular events are reminiscent of those involved in the neural mechanisms underlying learning and memory (Gunstream et al., 1995; Walters and Ambron, 1995; Kandel, 2001; Bailey et al., 2004). In addition, injury-induced neural plasticity shares common molecular mechanisms with nervous system responses to disease and degeneration (Wolpaw and Tennissen, 2001). These studies, as well as recent findings that have important implications regarding the regulation of gene expression during learning (Cohen-Armon et al., 2004), suggest that molecular mechanisms underlying learning might have evolved from the cell's response to stress or injury.

The aquatic worm, *Lumbriculus variegatus*, replaces lost body parts following amputation by activating several regenerative mechanisms (Drewes and Fourtner, 1990; Martinez et al., 2005b). Regeneration in *Lumbriculus* involves two developmental processes: epimorphosis and morphallaxis (Drewes and Fourtner, 1990; Martinez et al.,

2005b). Epimorphosis is the process underlying the replacement of lost body segments (Morgan, 1901). Morphallaxis is a form of regeneration that involves the reorganization or transformation of existing structures (Morgan, 1901). In regenerating worm fragments, neural morphallaxis is defined by transformation of the adult nervous system as the fragment acquires a new anterior-posterior positional identity and new neurobehavioral constraints (Drewes and Fourtner, 1990; Lesiuk and Drewes, 2001a; Martinez et al., 2005b). This rare form of neural plasticity is adaptive, since segmental regeneration is asymmetric. That is, fragments regenerate an eight segment head and a tail of variable length (Drewes and Fourtner, 1990; Lesiuk and Drewes, 2001a; Martinez et al., 2005b). As new head and tail buds develop, the original fragments typically experience a change in segmental identity as they acquire a more anterior body position. This change in positional identity, and the associated neural morphallaxis, involves the transformation of sensory, interneuronal, and motor pathways (Drewes and Fourtner, 1990; Martinez et al., 2005b). Neural morphallaxis is also activated prior to body fragmentation by architomic fission during asexual reproduction (Martinez et al., 2005b). Thus, like neural plasticity associated with adult learning and memory, neural morphallaxis likely involves cellular and molecular mechanisms that can be recruited to couple sensory inputs with long-lasting neurobehavioral changes in the absence of injury-induced signaling.

In the present study, we hypothesize that compromising the integrity of the central nervous system, either with extrinsic or intrinsic lesions which correlate with repair or reproduction is the initial trigger of neural morphallaxis. We have exploited the

unique regenerative potential of *Lumbriculus* to address this idea by experimentally inducing neural morphallaxis in posterior body segments including shifts in environmental condition and the induction of ectopic segmental regeneration. During the associated process of neural morphallaxis, the nervous system transforms its touch sensory fields, giant fiber conduction velocities, axonal diameters, and other physiological properties (Drewes and Fourtner, 1990; Martinez et al., 2005b). These cellular correlates of neural morphallaxis coincide with upregulation of a molecular marker of morphallaxis, a 60 KDa glycoprotein identified by a monoclonal antibody, Lan 3-2 (Martinez et al., 2005a). Since neural morphallaxis is correlated in time with these cellular and molecular events, the processes responsible for their induction can be elucidated.

Material and Methods

Animals and Maintenance

Worms were purchased from Flinn Scientific, Inc. (Batavia, IL). They were housed in moderately aerated spring water, at a constant temperature of 16oC ($\pm$1) in the dark. Worms were provided brown paper towel clippings for substrate and were fed spirulina powder and Tetramin staple flakes twice weekly.

To obtain experimental body fragments from specific body regions (anterior or posterior), worms were briefly anesthetized in 0.25 mM Nicotine in spring water. Segmental amputations were made at intersegmental boundaries with microdissecting scissors. Body fragments consisted of approximately 30 segments from the anterior

third of the worm and 30 segments from the posterior third of the worm. Regenerating body fragments were maintained individually in containers of spring water at 16^{o}C. Although animals regularly fragment by asexual fission in laboratory cultures, the rate of fission is depressed in animals maintained with sufficient aeration and substrate at cool temperatures. To promote asexual fission, worms were exposed to an environmental shift involving transfer to room temperature (22 ^{o}C) and culture conditions that lacked paper substrate and aeration for 3-4 days. Worms were then returned to cultures at 16^{o}C.

Ectopic Head Induction

Worms of 150 segments in length were partially transected on either the dorsal or ventral surface along the body axis of the worm. Worms were initially paralyzed in 0.25 mM nicotine solution and pinned onto sylgard blocks to immobilize the animal. Using vannas-nine microdissecting scissors, five segments worth of body wall, gut, and either dorsal blood vessel or ventral nerve cord was removed from these animals. These cuts were made at four segmental locations: segments 25-30; 50-55; 75-80; and 100-105. Animals were kept in plastic ice cube trays filled with Ozarka spring water following transection and were maintained with minimal disturbance and without contact with other worms at room temperature.

Boric Acid Treatment

Whole animals, or body fragments, were immersed in spring water containing boric acid (99.9% purity; 100 pM-50 mM) for 2 weeks. Animal survival and segmental regeneration was monitored daily. Newly regenerated head and tail buds were visually

inspected for the presence of defined segmental characteristics such as segmental boundaries and organization of the vasculature. Segments were counted as true segments based on the presence of defined intersegmental boundaries. Measurements of fragment weight, sensory fields, and giant fiber conduction velocities were calculated weekly. At the conclusion of exposure and testing periods, animals were prepared for protein extraction (see methods below).

Giant Fiber Sensory Field Mapping

Impulse conduction along giant nerve fibers was studied using non-invasive electrophysiological recordings (O'Gara et al., 1982; Drewes and Fourtner, 1990). Touch stimuli were delivered by a hand-held plastic probe. Medial giant fiber (MGF) and lateral giant fiber (LGF) action potential waveforms were distinguished based on previously reported spike characteristics (Drewes and Fourtner, 1989; 1990; Rogge and Drewes, 1993). Extracellular voltage recordings were obtained using a printed-circuit-board grid of electrodes and electrical signals were preamplified using a pair of differential recording amplifiers (100x gain, AC-coupled inputs). These spike recordings were digitized with a Powerlab A-D conversion system (ADInstruments, Inc.) and were analyzed on a G4 Macintosh computer (Apple, Inc.) using the Powerlab Chart software.

Spike conduction time between pairs of recording electrodes (5 mm pair spacing) was measured from peak-to-peak of giant fiber spike waveforms. Conduction velocity (m/s) was obtained by dividing the conduction distance by the spike conduction time. Individual means, based on 3-5 measurements per animal, were used in calculating group means. Non-invasive recording grids were also used to map giant fiber sensory

fields. Segments of specific identity (e.g., segment number 50) were marked with a spot of water-insoluble ink from a fine tip pen (Sharpie). Individual segments were then touched with a probe and giant fiber responses were monitored electrophysiologically.

SDS-Page and Western Blotting

Experimental fragments were cultured for 3 weeks post-amputation. Prior to homogenization, newly formed head and tail pieces where excised and discarded to remove factors specific to epimorphic tissues. Worm fragments were then homogenized in osmotic lysis buffer (10 mM Tris, pH 7.4 and 0.3% SDS) supplemented with a cocktail of protease inhibitors (20 mM AEBSF, 1 mg/ml leupeptin, 0.36 mg/ml E-64, 500 mM EDTA, 5.6 mg/ml benzamide) and nucleases (50 µg/ml RNase, 100 µg/ml DNase in 5mM $MgCl_2$ and 10mM Tris-Cl, pH 7.0). All steps of protein sample preparation were completed on ice. SDS-Page was performed according to standard procedures (Laemmli, 1970). Electroblot transfer was performed as in Towbin et al. (1979) using the Hoeffer system (Amersham) and electroblotting to 0.2 µm Nitrocellulose (Bio-Rad). Blots were incubated with diluted antibody (Lan 3-2, 1:10, J.Jøhansen; Anti-α-Tubulin, 1:1000, Sigma) and visualized using anti-mouse alkaline phosphatase conjugated secondary antibody (1:300; Vector). The signal was then developed with BCIP/NBT (tablets; Sigma). Stained gels or blots were digitized using a Nikon image capturing system and were analyzed using NIH Image densitometry analysis.

Statistics

Two-tailed student's t-tests (Microsoft Excel) or ANOVA (Statistica, Inc.) were used for statistical analysis. Data are presented as mean plus or minus standard deviation (s.d.) or standard error of the mean (s.e.m.) as indicated. Statistical significance was $p<0.05$.

Results

Ectopic Head Formation Depends on Nerve Cord Damage

Ablation of 5-8 segments of ventral nerve cord results in the formation of a ventrally protruding ectopic head in *Lumbriculus* (Lesiuk and Drewes, 2001b). However, the extent of damage necessary and sufficient to induce ectopic head formation is still unclear. We investigated the extent to which ventral nerve cord damage was necessary for ectopic head formation by creating three different populations of worms with varying levels of VNC damage. While each population demonstrated varying degrees of success at ectopic head formation, the structure of the ventrally protruding ectopic head was consistent with that of the original head with the exception, in some cases, of a reduction in the number of segments regenerated. Ectopic heads generally consisted of 5-8 body segments which projected ventrally to the longitudinal axis of the body (Fig. 17a). These heads consisted of a well-formed prostomium and demonstrated normal 'probing' and crawling activity. Removal of the original head following ectopic head formation also demonstrated that the ectopic head possessed the ability to function as the worm's primary head. Experimental populations consisted of

animals with either one, three, or five body segments worth of VNC and body wall removal in posterior segments. One and three segment VNC ablations resulted in the

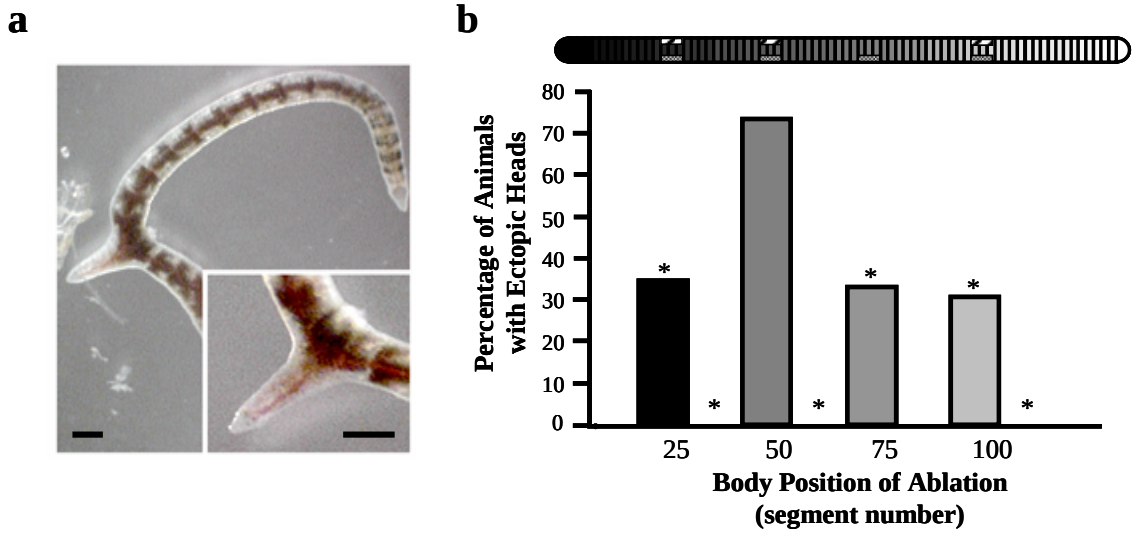

Figure 17 – Ectopic head formation requires VNC damage.

a. Ventrally protruding ectopic heads were produced following removal of 5 segments of ventral body wall and nerve cord. Ectopic heads generally contained 5-8 body segments with a well-defined prostomium (insert). Scale bar = 2μm.

b. Location and segmental position of the body lesion affect the percent of ectopic heads produced. Dorsally lesioned worms did not form ectopic heads irrespective of segmental position (n=121). The percentage of ectopic heads produced in ventrally lesioned worms varied with segmental position of the lesion. Specifically, 35% of worms with ventral ablations at segment 25 produced ectopic heads (n = 43); 71% at segment 50 (n = 23); 33% at segment 75 (n = 30); and 31% at segment 100 (n = 55). $P < 0.005$.

fewest ectopic heads produced: 7% produced with 1-2 segments of damage (n = 27) and 26% produced with 3-4 segments of damage (n = 27). Five to six segments of VNC damage produced the highest percentage of ectopic heads (40%; n = 37). Thus, nerve cord damage, ablation of 1-2 segments, was sufficient to induce ectopic head formation, but higher efficiency of ectopic head induction occurred with increased cord damage (5-6 segments ablation).

We have addressed the requirement for nervous system damage (i.e., VNC ablation) and the effect of damage location in ectopic head formation by setting up two experimental populations of worms. One population of worms was established in which 5 segments of dorsal body wall and gut was carefully removed, avoiding damage to the ventral nerve cord. A second population consisted of worms whose ventral nerve cord and body wall were removed. In each group, four segmental positions along the body of the worms (segment 25, 50, 75, and 100) were pre-selected as ablation sites. All worms injured on the dorsal surface failed to produce ectopic heads irrespective of segmental position of injury (n = 121; Fig. 17b). In contrast, ectopic heads were formed on worms with ventral nerve cord ablations at all sites tested along the animal's body (Fig. 17b). Interestingly, a significantly higher percentage of ectopic heads were produced between segments 50-55, a segmental position previously described as an area of sensory field overlap ($p < 0.005$; Martinez et. al., 2005b). Thus, damage to, and wound healing of, the body wall, dorsal blood vessel, and digestive tract are not sufficient to induce ectopic head formation. However, VNC damage may be necessary and sufficient to induce an ectopic head.

Neural Morphallaxis is Detected Following Ectopic Head Formation

Neural morphallactic reorganization of giant fiber anatomy and physiology occurred posterior to newly formed ectopic heads (Fig. 18). These morphallactic reorganization events are comparable to those which occur during segmental regeneration in amputated worm fragments and during asexual reproduction (Drewes and Fourtner, 1990; Martinez et. al., 2005b). To determine the extent of the

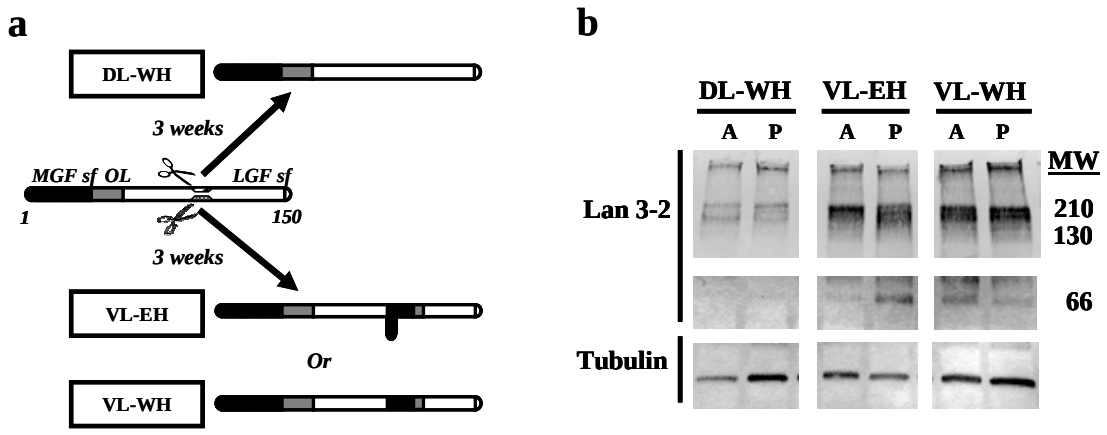

Figure 18 – Neural morphallaxis is detected posterior to the ectopic head.

a. Worms of approximately 150 segments were cut on either the dorsal or ventral surface. Five segments worth of body wall, gut, and in some cases ventral nerve cord, were removed. Dorsal and ventral ablations resulted in the formation of three populations of animals: dorsally lesioned animals which wound heal (DL-WH); ventrally lesioned animals with form ectopic heads (VL-EH); and ventrally lesioned animals which wound heal (VL-WH). Changes in giant fiber sensory fields were detected only in ventrally lesioned animals, irrespective of ectopic head formation. Specifically, medial giant fiber sensory fields (black shading) emerged in segments posterior to the ectopic head which originally only elicited lateral giant fiber sensory responses (white shading). Dorsally lesioned animals never exhibited changes in sensory fields. Areas of sensory field overlap are indicated by regions shaded in grey.

b. Lan 3-2 epitope expression was upregulated only in ventrally lesioned animals. Western blot analysis of protein extracts from dorsally lesioned animals (DL-WH) revealed protein profiles similar to those described in control (non-regenerating) worms. The 60KDa Lan 3-2 positive protein, a putative molecular marker of neural morphallaxis, is only upregulated in ventrally lesioned animals, especially posterior to the ectopic head (VL-EH; column P). Higher molecular weight proteins are also upregulated in ventrally lesioned worms. Tubulin antibody was utilized as a loading control.

effect of nervous tissue damage on neural morphallaxis we examined dorsally and ventrally lesioned worms for the emergence of medial giant fiber sensory fields posterior to the newly formed head or the wound site. Dorsally and ventrally lesioned worms resulted in three different outcomes: dorsally-lesioned worms that wound healed (DL-WH); ventrally-lesioned worms that wound healed (VL-WH); and ventrally-lesioned worms that formed ectopic heads (VL-EH). In dorsally-lesioned worms, tactile stimulation of the segments just posterior to the site of injury did not exhibit medial giant

fiber activity. Thus, the maps of medial giant fiber and lateral giant fiber touch sensory fields in segments near the site of injury appeared unchanged in comparison to normal worms (DL-WH; n = 12; Fig. 18a). Patterns of evoked medial giant fiber spiking in worms with ectopic heads (VL-EH) emerged just posterior to the newly formed head, an area that originally consisted of only a lateral giant fiber sensory field (VL-EH; n=4; Fig. 18a). Ventrally-lesioned worms which did not form an ectopic head but regenerated normally (VL-WH) demonstrated variable changes in touch sensory fields just posterior to the wound when measured three weeks following injury. Stimulation just behind the wound resulted in both MGF and LGF responses (NR; n = 6; Fig. 18a). This result is consistent with previous studies which describe wound healing and re-establishment of neural connections appropriately across the ablation site within 3 days of injury in these VL-WH worms (Lesiuk and Drewes, 2001b).

We have identified molecular markers of neural morphallaxis using a monoclonal antibody, Lan 3-2, that labels proteins possessing a mannosidic epitope. The expression of the Lan 3-2 epitope in ventrally-lesioned and dorsally-lesioned worms was examined. As previously described, multiple proteins that bear the Lan 3-2 epitope are induced during neural morphallaxis (Martinez, et. al., 2005a). In dorsally lesioned animals, Lan 3-2 positive proteins include a group of high molecular weight proteins at 210 and 130 KDa (DL-WH; Fig. 18b). However, the 60 KDa protein, which is upregulated during neural morphallaxis in regenerating fragments, was not detectable in dorsally lesioned animals. Interestingly, expression of higher molecular weight proteins

in the group DL-WH is identical to expression patterns previously described in control (non-regenerating) worms (Martinez et al., 2005a). In animals with ventral nerve cord ablations, there was significant upregulation of the higher molecular weight proteins (VL-EH and VL-WH; Fig. 18b). Moreover, there was marked upregulation of the 60 KDa protein just posterior to the newly formed ectopic head (VL-EH; column P; Fig. 18b). Interestingly, this 60 KDa protein was also upregulated both anterior and posterior to the ablation site in ventrally lesioned animals which regenerated normally (VL-WH; column A and P; Fig. 18b). These results are consistent with changes in giant fiber sensory fields in ventrally lesioned animals. Specifically, the upregulation of the 60 KDa proteins correlated with the emergence of medial giant fiber sensory fields posterior to the laterally protruding ectopic head (Fig. 18a).

Reduced Segmental Regeneration Does Not Affect Activation of Morphallactic Events

Changes in giant fiber sensory fields and upregulation of Lan 3-2 positive proteins in animals without ectopic heads (VL-WH), suggested that epimorphic regeneration of a new head may not have a direct influence on these neural morphallactic events. To test the extent of influence of epimorphic regeneration on neural morphallaxis, we measured cellular and molecular correlates of morphallaxis in worm fragments which exhibited a reduction in epimorphic regeneration. When applied during segmental regeneration of transected fragments, boric acid effectively delayed the formation of new head and tail segments but did not disrupt changes in giant fiber sensory fields or conduction velocity associated with neural morphallaxis (Martinez et al., 2005b). Regenerating fragments removed from the anterior 1/3 (AE) and posterior

2/3 (PE) body regions were treated with 10 mM boric acid and were processed for protein extraction at three weeks post amputation, a time point correlating to changes in cellular and molecular correlates of neural morphallaxis. Lan 3-2 epitope expression was not affected by boric acid treatment (BA+; Fig. 19a). Specifically, the 60 KDa Lan 3-2 positive protein continued to be upregulated in posterior fragments (PE) and was not significantly expressed in anterior fragments (AE).

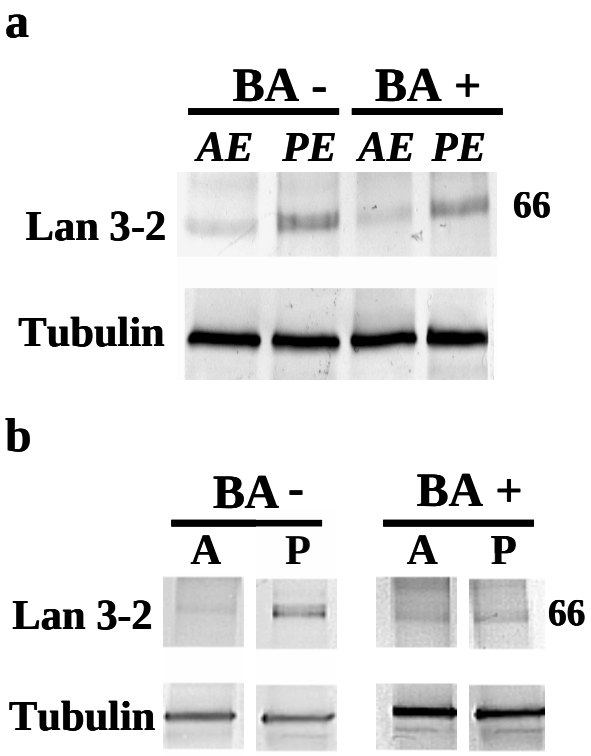

Figure 19 – Lan 3-2 epitope is not significantly affected by boric acid treatment.

a. Lan 3-2 expression was analyzed in worm fragments which were treated in 10mM boric acid to reduce segmental regeneration of a new head or tail. Upregulation of the 60KDa Lan 3-2 positive protein was not affected by boric acid treatment (BA+). Tubulin expression was also not affected by boric acid.

b. Boric acid treatment of asexually reproducing animals exhibited a slight decrease in the upregulation of the 60KDa glycoprotein. Tubulin was not affected.

Boric acid also blocks fission during asexual reproduction (Martinez et. al., 2005b). Protein extracts from asexually reproducing animals treated with boric acid were compared to those not treated. Although boric acid treatment did not affect changes in giant fiber anatomy or physiology (Martinez et al., 2005b), upregulation of the 60 KDa Lan 3-2 positive-protein posterior to the fission plane is slightly diminished (BA+; Fig. 19b).

Nerve Cord Injury Results in Abortion of On-going Asexual Reproduction

Lumbriculus reproduces asexually by a process called architomy. This type of asexual reproduction involves fragmentation at a predictable fission site, segment 48 ± 10 (in worms with 150 segments), resulting in two worm fragments (Martinez et al., 2005b). Interestingly, we observed that the formation of a fission plane in asexually reproducing animals was not indicative that the animals would complete this reproductive process. During experiments with asexually reproducing worms, a few individuals fragmented posterior to the fission plane and one day following fragmentation, previously formed fission planes were absent and fragmentation was aborted (Fig. 20a). Based on these results, we hypothesized that the process of regeneration in response to injury takes precedence over processes of plasticity associated with asexual reproduction.

Using non-invasive electrophysiological techniques, we probed segments posterior to the architomy site (segment 48 ± 10) for changes in giant fiber sensory fields. In control animals (normal asexual reproduction), medial giant fiber activity was recorded in segments posterior to the fission site, indicative that neural morphallaxis was

occuring normally (n = 27; Fig. 20a). However, experimental populations (aborted asexual reproduction) did not exhibit changes in giant fiber sensory fields behind the segments 48 ± 10 (defined architomy site). Specifically, medial giant fiber sensory fields were not recorded in segments posterior to the normal fission site (n = 27; Fig. 20a).

Protein extracts of both control and experimental groups were examined for upregulation of the Lan 3-2 epitope, a correlate of neural morphallaxis. In control worms, the Lan 3-2 epitope was highly upregulated in segments posterior to the fission site (Fig. 20b).

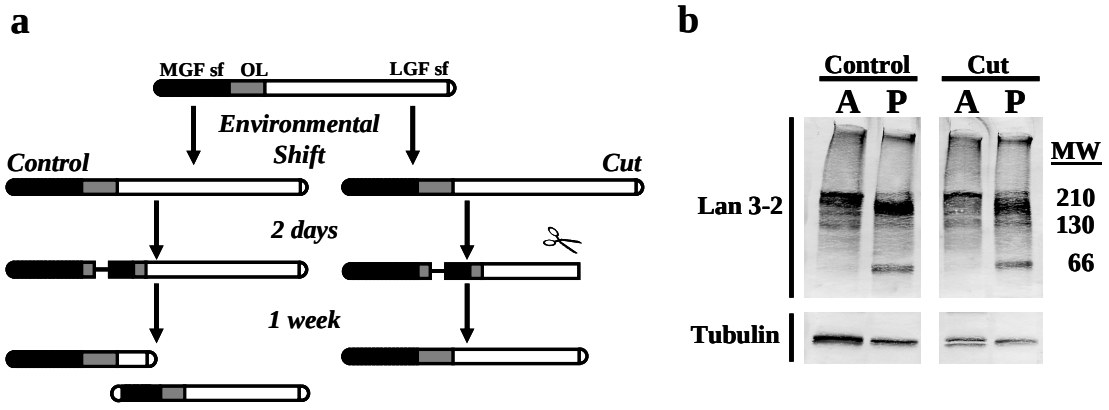

Figure 20 - Asexual fragmentation is aborted following posterior amputation.

a. Asexual reproduction was induced in whole animals using an environmental shift paradigm. Posterior segments (~ segments 100-150) of an experimental population of animals were removed two days following environmental shift. A population of worms which were not cut, continued through asexual fission normally and was used as a control. Worms which were posteriorly transected did not appear to complete asexual reproduction as fission planes were absent. Giant fiber sensory fields were also reversed in posteriorly cut animals. Medial giant fiber sensory field = black shading. Lateral giant fiber sensory field = white shading. Sensory field overlap = grey shading.

b. Immunoblot analysis of control (asexually reproducing) and cut (aborted asexual reproduction) animals. Protein extracts were created from segments anterior (A) to and posterior (P) to the fission site (48 ± 10) in control and cut animals. Upregulation of the 60 KDa Lan 3-2 positive proteins was detected in both control and cut animals. Tubulin was utilized as a loading control.

These results are consistent with the upregulation of the Lan 3-2 epitope in asexually reproducing animals prior to fragmentation (Martinez et al., 2005a). Surprisingly, Lan 3-2 upregulation was not detectably affected by the aborted asexual fission (Fig. 20b). Although a role for Lan 3-2 during neural morphallaxis is not established, these results suggest that Lan 3-2 upregulation precedes fission but is not sufficient for fission.

Discussion

Recovery from nerve injury involves the rapid initiation of processes that limit damage and subsequent mechanisms to rebuild them. In contrast to the limited recovery of function observed in the mammalian central nervous system (CNS) following injury, invertebrates can readily regenerate their nervous system and restore functionality with remarkable speed (Moffet, 1996). The nervous system is thought to play an important role in triggering some regenerative events in annelid worms (Drewes and Fourtner, 1991; Bely, 1999; Myohara et al., 1999; Yoshida-Noro et al., 2000). Our current studies demonstrate that compromising (lesioning) the nervous system is a critical event during the induction of neural morphallaxis, a unique form of neural plasticity associated with lumbriculid regeneration and reproduction.

Morphallactic reorganization within the worm's central nervous system has been suggested to result from morphogenic factors emanating from the newly regenerating head segments (Lesiuk and Drewes, 2001b). Utilizing a unique developmental paradigm which involves the formation of an ectopic head along the anterior-posterior axis of the worm, we have demonstrated that head formation (segmental regeneration) is not necessary for the induction of neural morphallaxis. Although ectopic head formation

was dependent on ablation of the ventral nerve cord, ventrally lesioned animals did not always produce ectopic heads. A small percentage of ventrally lesioned animals instead appeared to go through normal regenerative processes (VL-WH). Surprisingly, these VL-WH animals also exhibited cellular and molecular changes characteristic of neural morphallaxis. These results were consistent with previous studies which demonstrated the emergence of a reversal behavior (i.e., anterior-like behavioral responses) in ventrally lesioned worms three days following wound healing (Lesiuk and Drewes, 2001b). Furthermore, experiments using boric acid as an inhibitor of epimorphic regeneration (i.e. segmental regeneration) demonstrated that head regeneration, as a result of body transection or asexual reproduction, does not directly affect neural morphallaxis. These data suggest that formation of a regenerating head is sufficient for induction of neural morphallaxis but, perhaps not sufficient for maintenance. The distinction between an inductive and maintenance event is reminiscent of studies in *Aplysia* and the mammalian hippocampus which characterize learning and memory as a process involving multiple phases of events. Learning and memory mechanisms begin with inductive events which involve modification of pre-existing synapses and end with long-term synaptic changes which involve activation of gene expression and new protein synthesis for the stabilization of memory (Kandel, 2001; Bailey et al., 2004). Furthermore, it has been demonstrated that repair of an injured axon is initiated, maintained, and completed in a series of phases; the earliest phase composed primarily of the initial signals received by the nucleus following injury (Ambron et al., 1992; Dulin et al., 1995; Gunstream et al., 1995). Signals generated by axon injury then

trigger and prime transcription-dependent responses, such as growth and long-lasting compensatory changes in excitability (Ambron and Walters, 1996). Thus, it is interesting that, as described during learning and memory and axonal repair, the cellular and molecular events of neural morphallaxis can also be separated into phases which involve induction and long-term maintenance of cellular and molecular correlates.

Although VNC lesion alone is not sufficient to maintain neural morphallaxis, it may be necessary and sufficient for induction. Using dorsally and ventrally lesioned worms, we demonstrated that ventral nerve cord damage is sufficient for the induction of neural morphallaxis. Ectopic head formation was clearly dependent on VNC ablation, as none of the dorsally cut animals produced ectopic heads (Fig.17b). Moreover, the probability of ectopic head formation was increased with cuts made within segments 50-55 (Fig. 17b). These segments include those which comprise the site of fragmentation during asexual reproduction (Martinez et al., 2005b) and contain the region of medial giant fiber and lateral giant fiber sensory field overlap (Drewes and Fourtner, 1990; Martinez et al., 2005b). The high incidence of fragmentation at this site during asexual reproduction (Martinez et al., 2005b), together with high rates of ectopic head formation, suggests that this area of the nervous system is especially adapted for events of neural plasticity, akin to a developmental organizing center. Thus, it will be interesting to investigate further whether gradients of developmental signals that coordinate anterior-posterior identity can be functionally mapped to this region of the nerve cord. Based on our current data, we predict that this area may be endowed with attributes that confer a permissive state for plasticity.

Since neural morphallaxis can be initiated but is not necessarily maintained, like other forms of neural plasticity, this raised the possibility that once induced, neural morphallaxis could be aborted. Segmental regeneration following a complete transection appears to take precedence over on-going asexual reproductive processes. Asexual reproduction in *Lumbriculus* involves the intrinsic lesioning of the ventral nerve cord at a predictable fission site. In *Lumbriculus*, asexual reproduction results in the formation of two clones (zooids), worm fragments of approximately equal mass, that then individually go through epimorphic regeneration for the replacement of lost structures (Martinez et al., 2005b). This type of asexual reproduction is defined as architomy (Giese and Pearse, 1975). Using asexually reproducing worms we induced segmental regeneration by removing segments in the posterior body region (~ segment 100-150). Using this experimental paradigm, we demonstrated that the formation of the asexual fission plane is reversible. Furthermore, characteristic changes in giant fiber sensory fields of neural morphallaxis in segments near the fission site were aborted despite detectable Lan 3-2 upregulation. These results suggest the possibility that the distant influence of nerve injury, whether that of a morphogen or a neural signal, disrupts (inhibitory signal) cellular events which are required for the maintenance of neural morphallactic reorganization within the central nervous system. It is also possible that these data indicate a linear arrangement of the cellular and molecular correlates of neural morphallaxis during asexual reproduction, with the earliest events being Lan 3-2 upregulation followed by changes in cellular events and fragmentation.

In this study we utilized unique developmental paradigms which are reminiscent of early studies carried out with many invertebrates including hydra, snail, and annelids (Marcum et al., 1977; Bode and Bode, 1984; Vining and Drewes, 1985; Syed et al., 1992; Minobe et al., 1995) which addressed major questions of development such as that of polarity and regeneration. Regenerative studies in hydra identified molecules (morphogens) involved during regeneration and pattern formation in early development (Wolpert et al., 1972; Holstein et al., 2003). Head transplantation and ablation experiments in *Hydra* provided evidence for the existence of a head activator (HA) and a tail inhibitor, both produced by head tissue (Wolpert et al., 1972). These two factors were expressed in a gradient fashion with the highest amounts of both morphogens present in head regions. Similarly, ectopic head studies in *Lumbriculus* have provided possible evidence for morphogenic influences emanating from the regenerating head (Lesiuk and Drewes 2001b). The presence of Lan 3-2 positive proteins in both reproducing and regenerating animals without the regeneration of a new head or ectopic head suggests that there is an initial source of induction that is not found within the new head segments and that the regenerating head subsequently serves as a source of maintenance or amplification of the initial inductive signals.

We have also described evidence for the importance of neuronal and body injury on mechanisms of nerve regeneration. In other invertebrate and mammalian systems, nerve injury signals have been described to contain an important inductive role in nerve regeneration (Dinsmore and Mescher, 1998). In leech, it has been demonstrated that nerve injury induces a rapid efflux of nitric oxide (NO) which precedes the accumulation

of microglia at the site of lesion (Kumar et al., 2001). This NO-mediated accumulation of microglia at the lesion site subsequently aids in regeneration of the injured CNS axons through phagocytosis of debris and deposition of laminin to aid in axon guidance through the lesion (von Bernhardi and Muller, 1995). Nerve injury has also been shown to induce gap junctional coupling among adult motor neurons in the cat (Chang et al., 2000). Although, in the adult mammalian nervous system, neuronal gap junctional coupling is relatively rare, it is clear that injury-induced coupling between motor neurons may mediate signaling that maintains the viability of the axotomized connection until regenerated synapses are reestablished with their targets. Similarly, it is becoming clearer that stress (or noxious stimuli) is a biologically significant factor that, by altering brain properties, can disturb cognitive processes such as learning and memory (Kim and Yoon, 1998; Diamond et al., 1999; Kim and Diamond, 2002). It has been demonstrated in mammalian model systems that stress and stress hormones suppress induction of long-term potentiation (a sustained enhancement of synaptic efficacy that is thought to be the primary physiological model of memory) during hippocampal-dependent memory (Foy et al., 1987; Shors, et al. 1992; Kim and Yoon, 1998; Mesches et al., 1999). Additionally, recent studies in mice have demonstrated *in vivo* that a specific molecular event known to be initiated by stressful stimuli, also plays a role in forming long-term memory (Cohen-Armon, et al., 2004). These findings further strengthen the idea that molecular mechanisms underlying learning might have evolved from the cell's response to stress or injury (Walters, 1994). Thus, the demonstration that injury plays a role in neural morphallaxis implies an adaptive significance for the evolution of synaptic

plasticity mechanisms, like those of learning and memory, from primitive injury responses.

CHAPTER V

COMMON PROTEOMIC TRANSFORMATIONS DURING REGENERATIVE AND REPRODUCTIVE MORPHALLAXIS

Overview

Body fragments of the annelid worm, *Lumbriculus* variegatus, regenerate lost body parts during asexual reproduction and recovery from injury. This regeneration of new head or tail segments is accompanied by a transformation of the original fragments, a process termed Morphallaxis. Morphallaxis involves the reorganization of many body processes and structures, including the central nervous system, to match changes in segmental position as fragments regenerate. The cellular and molecular events that underpin neuro-behavioral plasticity associated with neural morphallaxis are largely unknown. Comparisons of proteomic profiles from 2D-SDS PAGE between control and regenerating fragments revealed an extensive percentage of proteins whose expression was altered during segmental position changes, such as the anteriorization of posterior fragments. However, only a small set of protein modifications was specifically correlated with neural morphallaxis. Additionally, proteomic profiles of asexually reproducing animals, which involved a more subtle change in fragment form and function, revealed less pervasive changes in protein expression. However, a small set of morphallaxis-specific protein changes was again upregulated in transforming segments. A morphallactic protein with a molecular weight of approximately 66 kDa (MP66) was upregulated in fragments of both regeneration and asexual reproduction groups. These

proteins are likely both MP66, since they shared common glycoepitopes and gel mobilities as indicated by western blot analysis.

Introduction

Regeneration is a trait represented among all metazoan groups (Sanchez-Alvarado 2000). However, although it is present in a variety of different species, there are examples within each phylum where this ability has been lost. For example, many annelids regenerate lost body segments, but significant variation in regenerative capacity exists among species. Many leeches have limited regenerative abilities, while some oligochaete worms can regenerate only posterior body parts (*Lumbricus terrestris*), and others possess far more extensive (*Lumbriculus variegatus*) regenerative ability (Berrill, 1952). Regeneration is defined by two mechanisms: epimorphosis and morphallaxis (Morgan, 1901). Each of these regenerative mechanisms has distinct cellular processes that mediate them. Epimorphosis typically involves the formation of a blastema followed by a period of growth and differentiation of missing body parts (e.g., amphibian limb bud). In contrast, morphallaxis involves the reorganization of existing tissues where cellular identities or functions are transformed without cell proliferation (e.g., planarian pharynx regeneration). Moreover, many animals that exhibit extensive regenerative capabilities also reproduce asexually (Berrill, 1952; Giese and Pierce, 1975). Asexual reproduction is thought to be related to regeneration in that each involves ‘development’ within an adult animal. Both asexual reproduction and regeneration involve processes that regulate axial patterning and plasticity. The nervous system changes underlying both asexual reproduction and segmental regeneration in

annelids are reminiscent of other types of adult neural plasticity, such as learning and memory. Therefore, we hypothesize that these two oligochaete life history events, asexual reproduction and segmental regeneration, share common molecular mechanisms.

Perhaps one of the most dynamic model systems of both morphallaxis and asexual reproduction is represented by the oligochaete worm, *Lumbriculus variegatus*. *Lumbriculus* regenerates an entire adult organism following amputative reduction to as little as three segments (Berrill, 1952). Worm fragments regenerate a new head and tail by forming buds via epimorphosis. At the same time, the original body segments producing the buds are reorganized by morphallaxis. *Lumbriculus* exhibits anterior-posterior gradients in anatomy, physiology, and behavior; all of which are altered during morphallaxis (Drewes and Fourtner, 1990). Moreover, head regeneration in *Lumbriculus* results in the production of a short 7-8 segment head (Berrill, 1952). Thus, changes in positional identity are especially evident in posterior fragments, which become more anteriorly located following head regeneration (Drewes and Fourtner, 1990). These posterior fragments undergo a sensory-motor reorganization, where tail-withdrawl responses present prior to injury are replaced by anterior behaviors (i.e., head withdrawl).

The reorganization of behavior during fragment regeneration observed in *Lumbriculus* is mediated by cellular and molecular changes within the CNS, a process called neural morphallaxis. This regenerative process is a novel form of neural plasticity defined by neuronal anatomical and physiological alterations (Drewes and Fourtner, 1990; Lesiuk and Drewes, 2001a; Martinez et al., 2005b). Furthermore, neural

morphallaxis is a developmental mechanism employed during both segmental regeneration (epimorphosis) and asexual reproduction by fission (architomy). During architomic fission, *Lumbriculus* fragments at a consistent fission zone at approximately segments 45-55 in worms of 150 segments (Martinez et al., 2005b). Neural morphallaxis occurs in segments just posterior to the fission site two days prior to fragmentation. Although neural morphallaxis is found in two developmental contexts which share some common mechanisms, little is understood about the cellular and molecular mechanisms which underlie neural morphallaxis.

Changes in gene expression that accompany neuronal regeneration have been identified (Welcher et al., 1991; Felipe and Hunt, 1994; Korneev, et al., 1997; Abankwa and Küry, 2004; Blackshaw et al., 2004; Wintzer et al., 2004). Gene expression profiling using microarray analysis of injured spinal cords (Abankwa and Küry, 2004; Bareyre and Schwab, 2003; Tanabe, et al., 2003) and subtractive cDNA libraries of regenerating and non-regenerating nerves (Korneev, et al., 1997; Blackshaw et al., 2004; Wintzer, et al., 2004) have identified genes whose expression is altered following neural injury. Some of these molecules are known to regulate aspects of neuronal development and thus are reactivated during regeneration of adult tissues (Jenkins et al., 1993; Zhou et al., 2004; Fernandes et al., 1999; Jung et al., 1997; Frey, 2000; Brecknell and Fawcett, 1996). Although these strategies have identified genes whose transcription is altered during regeneration, they do not reveal regenerative mechanisms involving regulation at translational or post-translational levels. We have employed proteomic analyses to

investigate changes in protein profiles associated with morphallaxis during segmental regeneration and asexual reproduction in *Lumbriculus*.

Morphallactic events in regenerating or reproducing worm fragments involve the induction of a 66 kDa protein (Martinez et al., 2005a). This 66 kDa protein is recognized by Lan 3-2, a monoclonal antibody which labels a mannosidic epitope born on proteins expressed in neural tissues within the CNS of *Lumbriculus* (Martinez et al., 2005a). In this study, we have analyzed changes in proteomic profiles of regenerating and asexually reproducing animals in an attempt to determine whether this 66 kDa protein is part of a set of morphallactic proteins shared by the mechanisms that govern both regeneration and asexual reproduction.

Materials and Methods

Animals and Maintenance

Worms were purchased from Flinn Scientific, Inc. (Batavia, IL). They were housed in moderately aerated spring water, at a constant temperature of 16^{o}C ($\pm$1) in the dark. Worms were provided brown paper towel clippings for substrate and were fed spirulina powder and Tetramin staple flakes twice weekly.

To obtain experimental body fragments from specific segmental regions (anterior or posterior), worms (~ 150 segments long) were briefly anesthetized in 0.25 mM nicotine in spring water. Segmental amputations were made at intersegmental boundaries with microdissecting scissors. Body fragments consisted of 30 segments (~ segments 8-38) from the anterior third of the worm and 30 segments (~ segments from

100-130) from the posterior third of the worm. Regenerating body fragments were maintained individually in containers of spring water at 16^{o}C.

Although animals regularly fragment by asexual fission in laboratory cultures, the rate of fission is depressed in animals maintained with sufficient aeration and substrate at cool temperatures. To promote asexual fission, worms were exposed to an environmental shift involving transfer to room temperature (22^{o}C) and culture conditions that lacked paper substrate and aeration for 3-4 days. Worms were then returned to cultures at 16^{o}C.

Two-Dimensional Gel Analysis

Two-dimensional gel electrophoresis was performed according to (O'Farrell, 1975) using the BioRad Model 175 Tube Cell. Regenerating fragments were cultured for 1-5 weeks post amputation. Newly formed head and tail pieces were removed to eliminate factors specific to epimorphic tissues (i.e., new segmental bud formation). Worms were homogenized in lysis buffer (25mM TrisBase, 2.5mM $MgCl_2$, 1.0mM EGTA, 8.0mM Urea, 1.0mM DTT, 1.0mM PMSF, 0.1% SDS, 1.0% Triton-X, 50 µg/ml RNase, 100 µg/ml DNase, 1 mg/ml leupeptin, 0.5 mg/ml aprotinin) on ice. Each sample contained 90 µg of total protein from control and experimental treatments. Samples were prepared in IEF sample buffer mix (9.9M urea, 0.3% SDS, 4%TritonX-100, 300mM DTT, 2.04 % Ampholines pH 5/7, 0.512% Ampholines pH 3.5/10) to a final volume of 20 µl. These IEF gels were run at 600 V for 16 hours. Second dimensional 15% acrylamide slab gels were run at 32.5 mA per gel for 6 hours using a Hoeffer SE600 15 cm gel system. Slab gels were fixed and silver stained according to a

modified Rabilloud protocol (Rabilloud, 1992). Stained gels were digitized using a Nikon image capturing system and were analyzed using NIH Image densitometry analysis.

Protein samples were also prepared from asexually reproducing animals. Fragments (~ 20 segments) were removed from 3 areas along the worm's body: anterior to the fission plane (~ segments 8-38); the zone of architomic fission (~segments 38-58); and posterior to the fission plane (~ segments 68-98). These worm fragments were then homogenized in osmotic lysis buffer (10 mM Tris, pH 7.4 and 0.3% SDS) supplemented with a cocktail of protease inhibitors (20 mM AEBSF, 1 mg/ml leupeptin, 0.36 mg/ml E-64, 500 mM EDTA, 5.6 mg/ml benzamide) and nucleases (50 µg/ml RNase, 100 µg/ml DNase in 5 mM $MgCl_2$ and 10 mM Tris-Cl, pH 7.0). Protein extracts were then quick frozen to aide in tissue disruption. SDS boiling buffer (5% SDS, 10% glycerol, 60mM Tris, pH 6.8) was added to each sample in equal amounts prior to boiling in a water bath for 30 min. Protein concentrations were determined following a quick centrifugation to rid the sample of lysed material. Each sample contained 65-68 µg of total protein. IEF gels were run as described above and 8% second dimension slab gels were used to increase resolution in the range of interest. Gels were either silver stained, prepared for electroblot transfer, or Coomassie stained and prepared for mass spectrometry. Silver stained gels were analyzed using PDQuest v7.3.1 software program (Bio-Rad). The analysis protocol included spot detection and filtering, background subtraction, spot matching, and volume analysis of each detected spot.

In-gel Digestion for Mass Spectrometry Analysis

Diced gel slices with desired protein spot(s) were reduced and alkylated (30% MeOH, 100 mM NH_4HCO_3; 2.5 mM DTT; 100 mM iodoacetamide). Proteins were extracted from polyacrylamide gel pieces using a modified Trypsin digestion (Stone and Williams, 1993). Briefly, spots of interest were excised and washed with 25 mM ammonium bicarbonate. The slices (or spots) were dehydrated with 50% acetonitrile and rehydrated with a solution of trypsin (20 ng/ul) in 25 mM ammonium bicarbonate. The protease was allowed to cleave at 37°C overnight. Peptides were extracted from the gel slices using 25 mM ammonium bicarbonate and subsequently purified using a ZipTip™ pipette tip (Millipore; Bedford, MA, USA).

Matrix-assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) and MS/MS Analysis

Purified protein samples were then analyzed on an Applied Biosystems Voyager STR MALDI-TOF mass spectrometer using alpha-cyano-cinnamic acid as a matrix. Additional analyses were performed on a ThermoFinnigan LCQ DecaXP ion trap mass spectrometer. Samples for MS/MS were separated on-line using a Surveyor HPLC system equipped with a Vydac C18 column (2185um315) attached directly to the source. Flow was split to approximately 20 nl/min. Peptides were separated using a gradient of acetonitrile from 5 to 50% over 50 minutes. Ions were analyzed using TurboSequest and BioWorks 3.0 and an EST database. The database included three annelids, the terrestrial oligochaetes, *Lumbricus rubellus* and *Eisenia foetida*, and a leech species *Hirudo*

medicinalis, that were downloaded from the National Center for Biotechnology Information (NCBI at http://www.ncbi.nlm.nih.gov/).

Two-Dimensional SDS PAGE Western Blotting

Two-dimensional gel analysis of extracted proteins was carried out using asexually reproducing worms (see above). Electroblot transfer was performed as in Towbin et al. (1979) using the Hoeffer system (Amersham) and electroblotting to 0.2 µm Nitrocellulose (Bio-Rad). Blots were incubated with diluted antibody (Lan 3-2, 1:10, provided by J.Jøhansen) and visualized using anti-mouse alkaline phosphatase-conjugated secondary antibody (1:300; Vector). The signal was then developed with BCIP/NBT (tablets; Sigma). Stained gels or blots were digitized using a Nikon image capturing system and were analyzed using Adobe Photoshop 6.0.

Results

Two-Dimensional Gel Analysis of Regenerating Fragments

Regenerating worm populations were produced by amputation and the removal of body fragments (~ 30 segments) from both anterior and posterior body regions (Fig. 21a). Because epimorphic regeneration of these fragments resulted in the production of short heads (7-8 segments), worm fragments obtained from posterior regions experienced a shift in positional identity (i.e., anteriorization) during regeneration. This change in segment positional identity is marked by a transformation of the central nervous system concomitant with changes in rapid escape reflexes (Drewes and Fourtner, 1990; Lesiuk and Drewes, 2001a; Martinez et al., 2005b). To examine

changes in proteomic profiles during morphallaxis, we analyzed protein extracts from both control (non-regenerating; newly amputated) and experimental (regenerating; amputated) worm fragments. Anterior worm fragments served as an important comparison group as they did not experience as dramatic a change in segmental position as posterior fragments following regeneration (Fig. 21a). Previous results demonstrated the upregulation of a 66 kDa protein marker of neural morphallaxis, termed here Morphallactic Protein 66 (MP66), at 3-5 weeks post-fragmentation, a time period associated with the most dramatic physiological and behavioral morphallactic changes (Martinez et al., 2005a). Thus, protein samples for two-dimensional gel analysis were extracted at 0 weeks (control fragments) and 4 weeks post-amputation (experimental fragments) to optimize differential profiling. Additionally, second dimension vertical slab gels were designed to ensure resolution of lower molecular weight proteins (i.e., 14.4 kDa to 97 kDa), a window that would included MP66. In fact, a doublet of proteins was present in posterior regenerating fragments that possess gel co-ordinates of approximately pI 8.5 and MW 66 (Fig. 21b). Thus, protein profiles compared here were representative of a sub-population of the total protein within body fragments.

Two-dimensional gels of control and regenerating fragments were analyzed using densitometry. Protein spots that surpassed a 5.5% normalized densitometry threshold (above background) were considered significantly expressed. Comparison of anterior and posterior protein extracts from control fragments revealed a greater abundance of detectable proteins in anterior segments (AC; Figure 22a). In these non-regenerating, anterior control segments, 129 proteins were detected above threshold; compared to

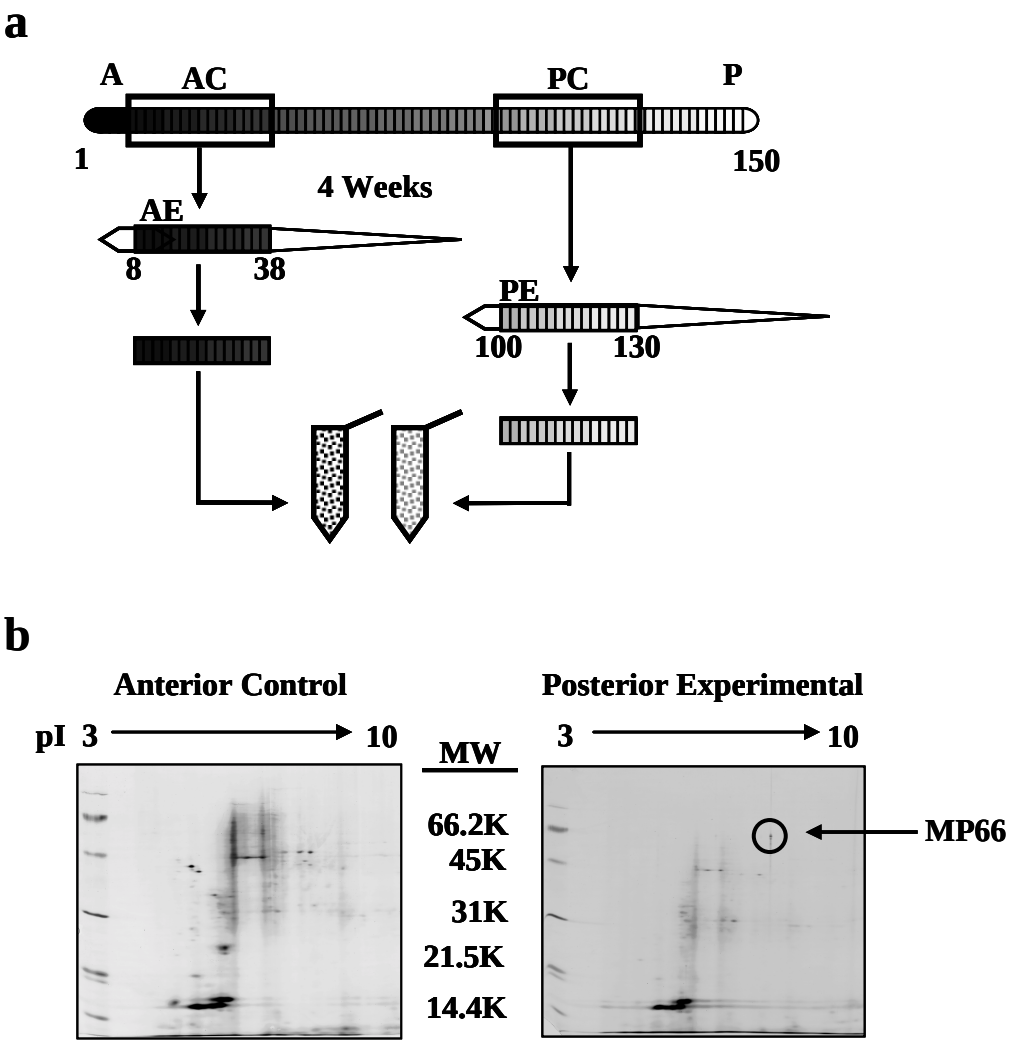

Figure 21 – Two dimensional gel analysis of regenerating worm fragments.

a. Whole animals of approximately 150 segments are used to create two regenerating (experimental) populations of fragments. Fragments (~ 30 segments) in length were removed from the anterior 1/3 and the posterior 2/3 body regions (AE and PE, respectively). Protein extracts of anterior (AC) and posterior (PC) control fragments were produced at 0 weeks of regeneration. Regenerating fragments were produced following 4 weeks of regeneration. At 4 weeks, newly formed head and tail segments were removed and discarded, leaving the original body segments for analysis. Protein extraction methods are found in the Methods section. A = anterior; P = posterior. Numbers indicate segment identity.

b. Protein extracts of anterior control and posterior experimental fragments were first isoelectrically focused using a pI range of 3-10 in the first dimension and were then run on a 10% vertical SDS PAGE gel to resolve low molecular weight proteins (~15-100 kDa). Determination of the numbers of proteins present was completed using densitometry analysis. Of the spots identified, several proteins were specific to posterior experimental fragments. A pair of robustly expressed proteins (circle) were detectable near a molecular weight of 66 kDa (~ pI = 8.5). One of these is likely MP66. Gels were silver stained.

only 51 detectable proteins in non-regenerating, posterior control segments (PC; Figure 22a). Comparisons of proteins present in both control and regenerating fragments revealed significant changes in the relative expression levels of many proteins in posterior fragments during morphallaxis. Figure 22b illustrates that AE segments possessed few proteins whose expression levels were greater than 40% higher than corresponding proteins in AC segments. On the contrary, 80% of the proteins expressed in both PE and PC segments exhibited proteins levels greater than 40% in PE segments. Interestingly, approximately 40% of the proteins shared by AC and AE segments were expressed at substantially lower levels in AE segments. No proteins were expressed at high levels in PC segments compared to PE segments. Thus, of the proteins that were detectable in both control and experimental segments, a large portion were up-regulated in posterior fragments during regeneration and, to lesser extent, a portion was down-regulated in anterior fragments during regeneration. Thus, an anterior-posterior gradient in the levels of detectable proteins existed in control animals, with fewer proteins attaining the threshold level of significant expression in posterior segments. The observation that over the time course of regeneration (4 weeks), AE fragment protein numbers declined and PE fragment protein levels increased, suggested that these proteomic transformations reflect actual changes in protein expression associated with segment posteriorization and anteriorization rather than artifacts of protein extraction or resolution. Furthermore, of the 132 proteins compared across fragment populations, only 2 were specific to posterior experimental fragments (Figure 22a). One of these

proteins was MP66 as determined by its cross-reactivity with an antibody that labels a glycoepitope associated with MP66 (Martinez, et al., 2005a).

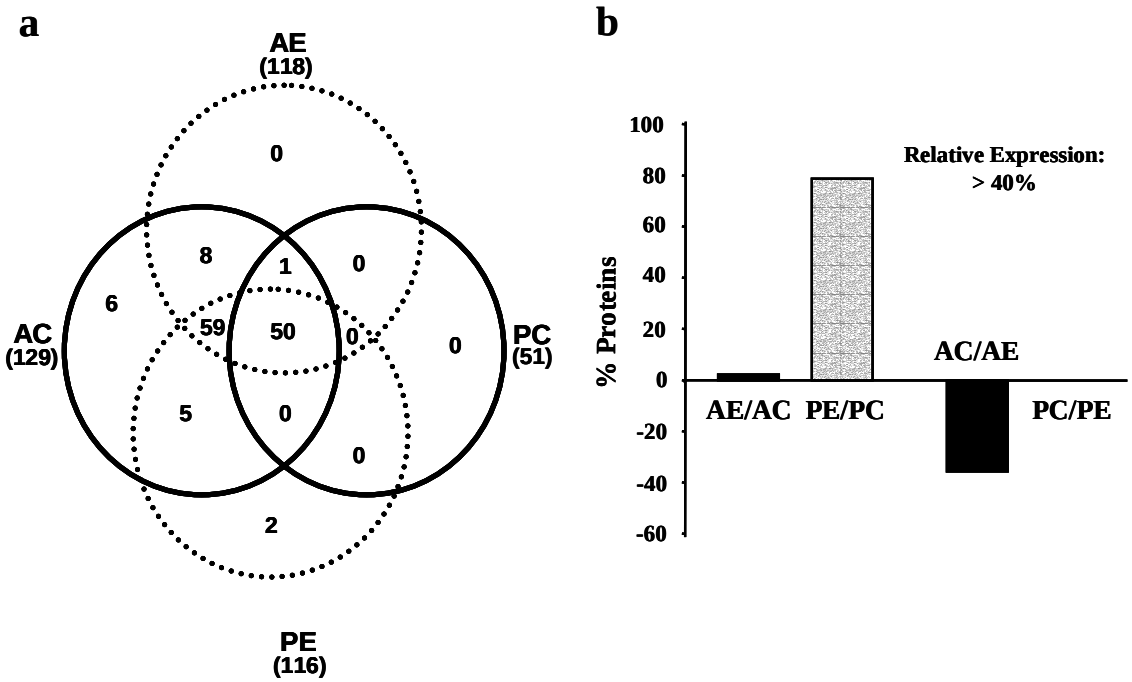

Figure 22 – Proteomic profiles of control and regenerating worm fragments.

a. Venn diagram indicating the number of significantly expressed proteins detected in anterior or posterior fragments using analysis of 2-D gels. Comparisons of anterior and posterior control fragments show a greater abundance of detectable proteins in anterior fragments (AC;PC). Posterior regenerating fragments (PE) possessed a significantly greater number of proteins than posterior controls (PC). The number of detectable proteins in anterior segments was greater than in anterior regenerating fragments (AE). These changes in numbers of significantly expressed proteins posterior fragments included 59 proteins common to AC and PC fragments. Only two PE proteins were specific to those fragments (i.e., not detectable in AC, AE, or PC group). No proteins were AE-specific. Numbers in parenthesis are total numbers of proteins detected.

b. Histogram of the % proteins that were highly up- or down-regulated during neural morphallaxis. Comparisons of anterior regenerating (AE) and posterior regenerating fragments (PE) and their respective controls (AC & PC) indicated that not many anterior proteins were significantly upregulated (AE/AC) during regeneration. However, posterior regenerating fragments possessed many proteins that were highly upregulated during neural morphallaxis (PE/PC). A few anterior proteins were down-regulated in regenerating fragments (AC/AE,) but no proteins were detectably down-regulated in posterior fragments (PC/PE).

Two Dimensional Gel Analysis of Asexually Reproducing Animals

We hypothesized that PE specific proteins, including MP66, represent molecules specifically induced during neural morphallaxis and therefore, would be upregulated

during fragmentation associated with asexual reproduction. To test this idea, we analyzed changes in protein profiles of asexually reproducing worms, a behavioral context that involves neural morphallaxis and the recruitment of developmental events, but does not involve injury or wound healing. Thus, upregulation of proteins in this context would not be the result of injury-specific processes. Protein extracts were collected two weeks post-induction of asexual reproduction, a time corresponding to cellular and molecular changes associated with neural morphallaxis. These extracts were produced from segments removed from three body regions: before, at, and behind the architomic fission zone (Figure 23a). Isoelectric focusing (IEF) gels of the asexual proteins were run on low percentage (8%) acrylamide slab gels which allowed for higher resolution of proteins found within the 40 - 80 kDa range. Two proteins were highly expressed in middle and posterior segments (circle; Figure 23b), but not in segments anterior to the nascent fission zone. Likely, one of these proteins corresponded to MP66, as its gel coordinates were virtually identical to those of regenerating fragments (pI 8.5; MW 66 kDa). Protein spots that surpassed a 6 % normalized densitometry threshold (above background) were considered significantly expressed proteins. Like control fragments in the amputation studies, a higher abundance of proteins were detected in anterior segments (128 protein spots) as compared to 120 proteins detected in middle segments encompassing the fission zone and 104 proteins in segments more posterior (Figure 24a).

Comparisons of proteins present in worm segments from control and asexually-reproducing worms revealed significant differences in the relative expression levels of many proteins. Figure 24b illustrates that greater than 90% of proteins common to both

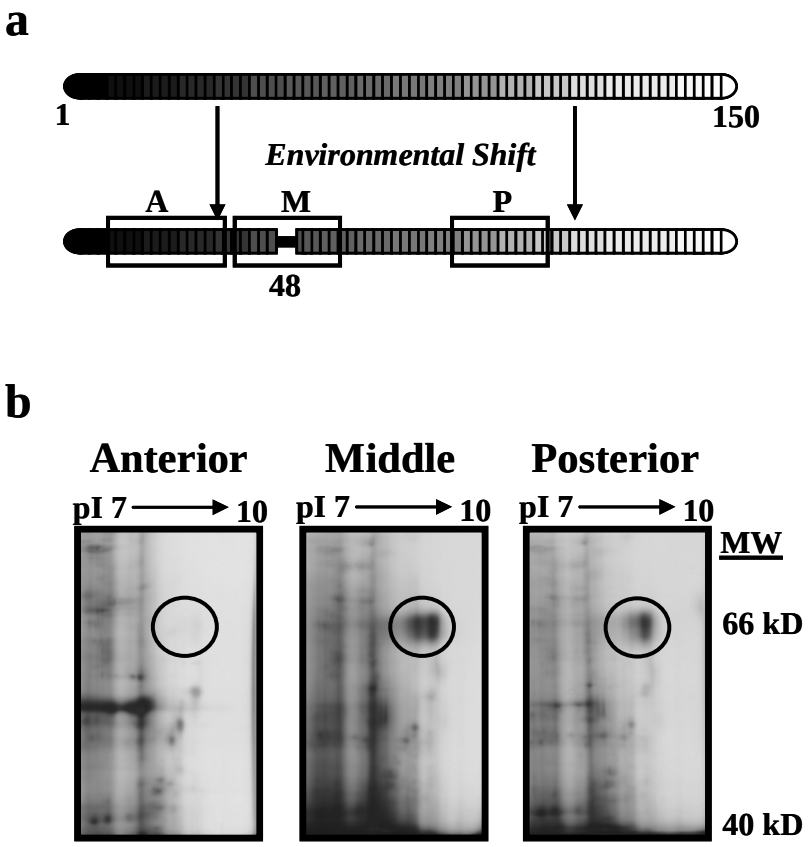

Figure 23- Two-dimensional gel analysis of asexually reproducing worms.

a. Asexual reproduction was induced in animals of approximately 150 segments using an environmental shift assay (see Methods). Most animals initiate the formation of architomic fission planes at segment 48±10 within two weeks of the shift. At this time, two weeks post-environmental shift, protein extracts were produced from three body regions: anterior to the fission zone (A); at the fission zone (M); and posterior to the fission zone (P).

b. Protein extracts of asexually reproducing worms were separated using isoelectric focusing at a pI range of 3-10. These gels were then run on an 8% acrylamide gel to allow for higher resolution of 40-80 kDa proteins. Densitometry analysis revealed that a 66 kDa protein is highly expressed in middle and posterior segments and is not found in anterior segments (circles).

anterior and posterior segments were expressed at levels 40% greater in anterior segments than posterior segments. Essentially no proteins were expressed at

substantially higher levels in posterior segments than anterior. However, following the induction of asexual reproduction, the expression levels of many fewer proteins were markedly higher in anterior segments compared to posterior. In contrast, several weeks

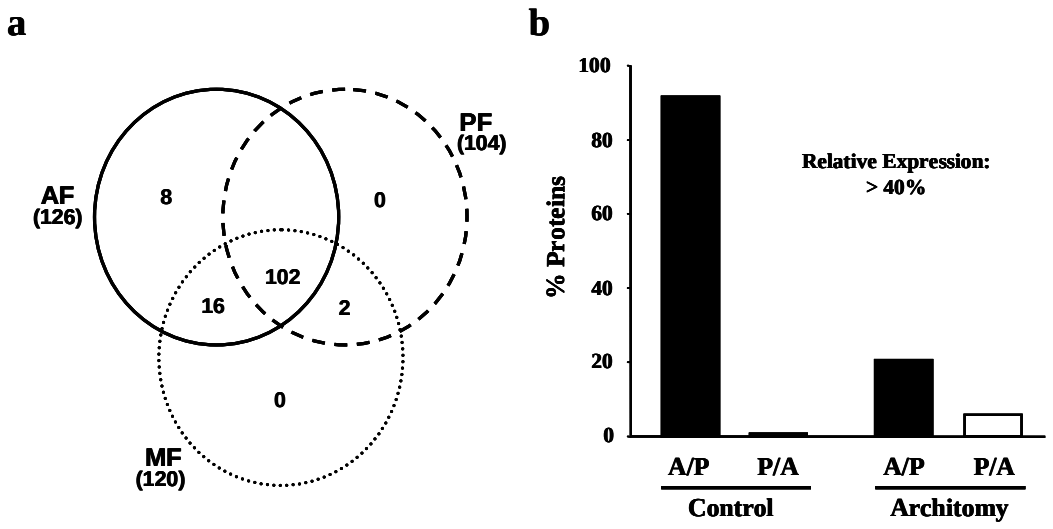

Figure 24 – Proteomic profiles of asexually reproducing animals.

a. Venn diagram illustrates the number of proteins detected that were expressed 6% above background levels using densitometry of 2-D gels. Segments anterior to the fission zone (AF) possessed a higher abundance of proteins as compared to middle segments (MF) or posterior segments (PF), at and behind the fission zone, respectively. Two proteins were robustly expressed in both middle and posterior segments but not in anterior segments. Eight proteins were anterior-specific. Numbers in parenthesis are total numbers of proteins detected.

b. Histogram of percent proteins that were highly expressed in anterior and posterior regions during asexual reproduction. A gradient in the number of proteins expressed was exhibited in control worms. Almost 100% of all proteins expressed in anterior segments were present in substantially higher abundance in anterior versus posterior segments (A/P). Only 1% of proteins in posterior segments were significantly higher in abundance than compared to their expression in anterior segments (P/C). During asexual reproduction, the percent of proteins that were highly expressed was greatly diminished (A/P-Architomy vs. A/P-Control). During architomic reproduction, more proteins exhibited significantly greater levels of expression in posterior segments compared to anterior segments (P/A). In contrast, fewer proteins in anterior regions exhibited markedly higher anterior versus posterior expression (A/P).

after induction of reproduction, several more proteins were now more highly expressed (> 40% higher) in posterior segments than in anterior body regions. One of these upregulated proteins corresponded to MP66. Proteomic analyses of middle body segments, encompassing the architomic fission zone, also revealed marked upregulation

of protein expression in this body region, including the expression of MP66 (data not shown). Thus, of the proteins that were detectable along the anterior-posterior body axis of *Lumbriculus*, a large portion were up-regulated in middle and posterior segments during asexual (architomic) fission.

Lan 3-2 Epitopes Are Detected on Proteins Upregulated During Asexual Reproduction

MP66 is a Lan 3-2 positive-protein, meaning it bears a mannosidic epitope that cross-reacts with a specific leech monoclonal antibody. MP66 (or the Lan 3-2 epitope) was differentially upregulated along the anterior-posterior axis of asexually reproducing animals, with intense expression detected in segments at the fission site (Mid) and a slightly decreased expression in segments posterior to the fission zone (Post; Figure 25a). Higher molecular weight Lan 3-2 positive-proteins were detected in anterior segments, however, as in 2-D gels; MP66 was not highly expressed in anterior segments (Ant; Figure 25a).

Two-dimensional western blot analysis was performed on asexually reproducing worms to determine if any significantly expressed proteins were Lan 3-2 positive. A Lan 3-2 positive protein of 66 kDa was highly expressed in segments associated with the fission zone (Middle) and in segments posterior to the zone (Post; Figure 25b). Interestingly, western blots indicate several protein spots at the MP66 coordinates (pI 8.5; MW 66 kDa) with variable levels of intensity, perhaps indicative of variable glycosylation with the Lan 3-2 epitope. Higher molecular weight proteins were also labeled with the Lan 3-2 antibody in segments anterior to the fission site (data not

shown); which correlated with expression patterns obtained using one-dimensional immunoblot detection (Figure 25a).

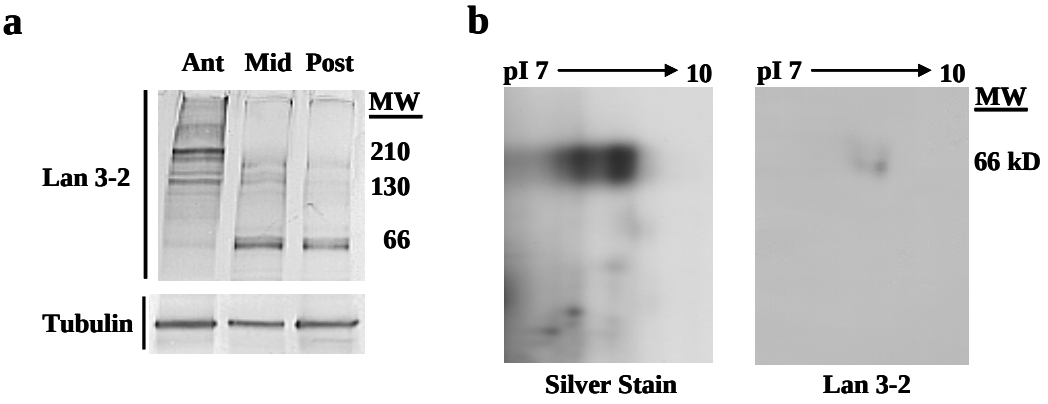

Figure 25 – Western blot analysis of asexually reproducing worms.

a. Immunoblot analysis of asexually reproducing worms using the Lan 3-2 monoclonal antibody. One-dimensional gel analysis of proteins revealed that higher molecular weight Lan 3-2-positive-proteins (210 & 130 kDa) were highly expressed in anterior segments and were expressed at much lower levels in middle and posterior segments. A 66 kDa protein was significantly expressed in middle and posterior segments, but was only weakly detected in anterior segments. Tubulin was utilized as a loading control.

b. Two-dimensional gel western blot analysis demonstrates that the 66 kDa protein, which was highly expressed in middle and posterior segments, was a Lan 3-2 positive-protein. Although the protein staining appeared as a large streak in the silver stained gel (left panel), the Lan 3-2 staining of the western (right panel) indicated the presence of multiple protein spots.

Mass Spectrometry Analysis Reveals a Mass Fingerprint of Proteins That May Be Involved During Morphallaxis

Mass spectrometric data collected for MP66, isolated and purified from 2-D gels, was compared to expressed sequence tag (EST) databases composed of multiple oligochaete EST libraries, including *Lumbricus rubellus*, *Eisenia foetida*, and *Hirudo medicinalis*. No *Lumbriculus* library currently exists. The experimental masses (M_r Obs.) of protein fragments and estimated protein sequences generated using mass spectrometry did not conclusively match to any known protein. Comparisons did

produce several identified (Table 2) and non-identified protein products (Appendix B) which were selected based on correlation values (XC) greater than 1. Interestingly of

Table 2 - Mass Spectrometry Data: Comparison to Known Proteins

Protein	Accession No.	Sequence	M_rTh./Obs. (kDa)	XC> 1
receptor tyrosine phosphatase [Hirudo medicinalis]	T30938			
Scan No.196		*R.NFILKCSATGDPEP SVYWLK.D*	2268.61 /2268.1375	1.29
Scan No.198		*K.FIDGITGPPYQLR.V*	1476.70 /1476.7851	1.12
netrin precursor [Hirudo medicinalis]	AAC83376			
Scan No.187		*K.IYNKGPR.G*	846.98 /847.479	1.16

the ESTs searched, the leech receptor tyrosine phosphatase and leech netrin displayed significant similarities to the MP66 protein (Table 2). Further analysis using MALDI-TOF mass spectrometry and Edmund's N-terminal sequencing is needed for future identification of the MP66 protein.

Discussion

Neural plasticity refers to dynamic changes in the form and function of a nervous system during particular events such as development and learning. Many neurons exhibit plasticity; that is, they can change structurally or functionally, in both short- or long-lasting manners. Neural plasticity exists, not only in development and learning, but

in diverse phenomena, including processes such as memory, drug tolerance, and recovery of function after lesion (Baudry, et al., 1999). Neural morphallaxis in *Lumbriculus* provides a unique platform for the study of neuronal plasticity. Morphallaxis involves changes within the nervous system that accommodate the new positional identity of the system. This morphallactic reorganization of the nervous system is essential to the functional regeneration of rapid escape reflex behaviors, which are specific to each body region. While these dynamic reorganizational events likely involve a broad range of proteins, comparisons of protein profiles of regenerating and non-regenerating worm fragments have indicated a surprisingly small subset of proteins which are differentially expressed and are specific to tissues undergoing morphallaxis. Given that invertebrate animals, like *C. elegans* and *Drosophila*, contain proteomes of about 20,000 and 14,000 proteins respectively (Harrison et al., 2002), we can predict that the window of proteins we analyzed in regenerating body segments of *Lumbriculus* might cover 0.6 – 0.9 % of the total lumbriculid proteome. Since approximately 5% of the proteome examined here was transformed in a morphallaxis-specific manner, we predict that, assuming a total proteome of 14,000 proteins, some 700 proteins could mediate the process of morphallaxis in *Lumbriculus*. Of these potential morphallaxis-specific proteins, MP66 was repeatedly upregulated in tissue experiencing dramatic morphallactic changes during both segmental regeneration induced by injury and the intrinsically regulated process of asexual reproduction.

MP66 is detected in both regenerating and asexually reproducing animals. During regeneration following amputation, MP66 is expressed 3-5 weeks post-injury in

posterior segments which are experiencing anteriorization. This time point correlates with most of the cellular changes associated with neural morphallaxis (Drewes and Fourtner, 1990; Lesiuk and Drewes, 2001a; Martinez et al., 2005a; b). Moreover, the expression levels of MP66 are highly induced in middle segments (segments 38-58) during asexual reproduction. These segments are also characterized by an overlap in giant fiber sensory fields, the sensory-interneuron pathways that mediate rapid escape reflexes, (Drewes and Fourtner, 1990) and are the segments which make up the site of fission during asexual reproduction (Martinez et al., 2005b). This sharp upregulation of MP66 occurs one week prior to fragmentation, paralleling cellular reorganization events, in asexually regenerating worms (Martinez et al., 2005b). Thus, the expression pattern of MP66 suggests that it is a molecular marker specific to tissues undergoing morphallaxis.

Studies of regeneration in classic model systems have identified gradients in the expression of molecules (morphogens) that set up anterior-posterior boundaries that organize the replacement of missing body parts (Holstein et al., 2003). It is thus hypothesized that specific regions of this adult tissue contain an “organizer-like” function, which reestablishes these gradients during regenerative processes. Interestingly, protein gradients are also detected in *Lumbriculus* along its anterior-posterior axis. Lan 3-2 specific proteins, including MP66, are differentially expressed in anterior and posterior control animals, with higher molecular weight proteins being more prevalent in anterior segments and lower molecular weight proteins in posterior segments (Martinez et al., 2005a). Thus, it appears that a gradient of molecular

signaling may play a role in lumbriculid morphallaxis. Moreover, the middle segments of the worm, which is the center for many important cellular events during morphallaxis, are highly specialized for neural plasticity and as a result may serve as an organizing center of instructive signals utilized during morphallaxis in *Lumbriculus*.

A long-standing question of neuroscience has been to understand the cellular and molecular mechanisms which underlie neural regeneration. The answer to this question has been confounded by the lack of genetic tools in classic regenerative model systems. Although little is known of the global genetic or proteomic changes involved during mammalian CNS regeneration (Wintzer et al., 2004), it is not surprising that studies of hydrozoan and planarian regeneration indicate that some genes involved in embryogenesis also act during regeneration (Hobmayer et al., 2000; Sanchez-Alvarado and Newmark, 1998; 1999; Agata and Wantanabe, 1999). In *Lumbriculus*, Lan 3-2 positive proteins label collateral branches, sites of synaptic input to and output from the central nervous system (Martinez et al., 2005a). Thus, we have hypothesized that the upregulation of Lan 3-2 positive-proteins, for example MP66, may underlie changes in synaptic connectivity responsible for changes in neuronal network physiology during neural morphallaxis. The Lan 3-2 antibody labels a mannosidic epitope found on leech sensory neurons during neural development (Zipser and McKay, 1981). Using antibody perturbation experiments, the Lan 3-2 mannosidic epitope was demonstrated to mediate the defasciculation, sprouting, and arborization of sensory neuronal processes as they enter the CNS (Briggs et al., 1993; Zipser et al., 1994; Song and Zipser, 1995; Zipser, 1995). Thus, it is possible that Lumbriculid morphallactic proteins, which are also Lan

3-2 positive, are composed of protein families that play important roles in neural development, specifically during axonal outgrowth, target selection, and synapse formation. The expression of Lan 3-2 positive-proteins in *Lumbriculus* embryos remains unstudied.

Although mass spectrometry analysis of lumbriculid protein extracts did not allow identification of the proteins upregulated during neural morphallaxis, two leech proteins, a receptor tyrosine phosphatase (HmLar) and netrin, were among the list of candidate proteins which demonstrated mass spectrometric similarities to MP66. Members of the large family of protein tyrosine phosphatases (PTPs) have been implicated in a wide range of physiological processes including cell adhesion, cell migration, and development of the immune and nervous systems. Recently, receptor protein tyrosine phosphatases (RPTPs) have emerged as key regulators of axon growth and guidance (Baker and Macagno, 2000a; 2000b; Stoker, 2001; Johnson et al., 2001; Biswas, et al., 2002; Johnson and Van Vactor, 2003). It is intriguing that the sequence from HmLar (Gershon et al., 1998) with similarity to sequences generated of MP66 corresponds to a highly conserved portion of a HmLar extracellular Ig domain (Table1). The Ig superfamily of proteins is largely made up of cell adhesion molecules (Grenningloh et al., 1990; Rathjen and Jessell, 1991); including CAMs that orchestrate the highly specific patterning of neural connections during development. CAMs promote neurite outgrowth of specific neuronal populations and organize similar axons into coherent fasciculated projections (Goodman and Shatz, 1993; Johansen and Johansen, 1997). Moreover, two neural cell adhesion proteins, LeechCAM and Tractin,

possess the Lan 3-2 epitope and label neural pathways in *Hirudo medicinalis* and *Haemopis marmorta* (Huang et al, 1997; Jie et al., 1999).

RPTPs are themselves a part of the Ig superfamily and are known to interact with other CAMs (Huber, 2003). In *Drosophila*, loss of Dlar function also results in reduced synapse size and decreased terminal branch complexity (Kaufmann, et al., 2002; Broadie and Richmond, 2002). RPTPs' role during regeneration is especially intriguing in light of recent studies which describe delayed peripheral sciatic nerve regeneration and a significant decrease in central nervous system collateral sprouting in LAR-mutant mice (Xie et al., 2001;Van der Zee et al., 2003). It is therefore, interesting that RPTPs or another member of the Ig superfamily may also be involved during *Lumbriculus* neural morphallaxis.

CHAPTER VI

GENERAL CONCLUSION

Since T.H. Morgan's description of morphallaxis in the early 1900s, few studies have attempted to unravel the mechanisms behind this regenerative process. While morphallaxis has been described in a few animal groups, including cnidarians (Holstein et al., 2003), flatworms (Reddien and Sanchez-Alvarado, 2004), and annelid worms (Myohara, 2004) and tunicates (Kawamura and Fujiwara, 1995), many more studies have focused on the mechanisms involved in epimorphic regeneration, perhaps because this type of regeneration is more common in vertebrate species (Slack, 2003; Chernoff, et al., 2003). The elegant studies of morphallaxis preformed by Drewes and Fourtner (1990) on the oligochaete worm, *Lumbriculus variegatus*, defined this model system as a unique tool for the study of morphallaxis within the nervous system (neural morphallaxis). Still even, in the light of this system's novelty and potential, only a few studies of *Lumbriculus* morphallaxis have been reported in the last 15 years. The present work on this model system has largely derived from tests of the general ideal that morphallaxis involves the redeployment of developmental programs in an adult animal and demonstrates that cellular and molecular mechanisms of neural morphallaxis are recruited during both injury-induced regeneration and asexual reproduction. These studies establish that the developmental events underlying neural morphallaxis can be induced in response to segmental injury (fragmentation by amputation) or prior to

fragmentation during the formation of architomic fission zones that precede asexual reproduction (Chapter II). Experiments using boric acid (Chapters II and IV), a chemical treatment that inhibits the epimorphic regeneration of head and tail buds, did not disrupt the recruitment of morphallactic regeneration. Additionally, boric acid-mediated elimination of fission plane formation had no affect on the induction of morphallaxis in animals placed in conditions that elicit asexual reproduction.

Results of boric acid experiments demonstrate two fundamental features of neural morphallaxis in *Lumbriculus*. First, on-going segment replacement (epimorphic growth) is not a prerequisite for the determination of new axis positional identity, as indicated by the induction of morphallactic transformation. Second, developmental signaling mechanisms and cellular differentiation events, associated with architomic fission plane generation, are functionally separable from the cellular and molecular events that govern morphallactic regeneration. One interpretation of these results is that epimorphic, architomic and morphallactic signaling cascades involve independent control mechanisms (e.g., morphogenic induction, gene activation, enzymatic activity, etc.). Thus, in the absence of the normal linkages between developmental pathways that govern an integrated biological event (e.g., reproduction), component cascades can endure, while others fail.

Although the molecular mechanisms of neural morphallaxis in *Lumbriculus* are unknown, the present proteomic studies (Chapter V) demonstrate that the cellular and behavioral correlates of neural morphallaxis are accompanied by a significant upregulation of the *Lumbriculus* proteome, including the induction of Lan 3-2

immunoreactive proteins. Comparative proteomic analyses indicate that one of these molecules, a 66 kDa glycoprotein, is part of a cassette of molecules specifically correlated with morphallactic transformations, during both recovery from injury and in advance of architomic fragmentation (Chapters III and V). Therefore, this morphallaxis-associated protein has been identified as MP66 documented here. Whether the upregulation of MP66 involves posttranslational events responsible for increased protein glycosylation with the Lan 3-2 epitope, or actual elevations in the expression levels of the protein itself, has not yet been distinguished. Nonetheless, carbohydrate recognition has long been established as an important role in the patterning of neural networks during development (Jessell et al., 1990; Nguyen, et al., 2003). Specifically, interactions between cell surface oligosaccharides and carbohydrate-binding proteins regulate many aspects of axonal outgrowth and synaptic targeting (Jessell et al., 1990). Moreover, antibody perturbation experiments in leech have demonstrated that the Lan 3-2 epitope is necessary for defasciculation of sensory afferents during leech synaptogenesis (Zipser and Cole, 1991); and therefore is an integral element in the embryonic process of constructing this annelid's nervous system. Alterations in the abundance of this specific glycoepitope, or the proteins that bear it, would undoubtedly have a significant impact on carbohydrate signaling during neural morphallaxis, and could potentially mediate changes in synaptic connectivity that accompany neurobehavioral plasticity, similar to this epitope's role in leech nervous system development.

MP66 induction is common to both asexually reproducing and segmentally regenerating animals. Immunocytochemical and biochemical studies presented here

demonstrate that Lan3-2 epitope-bearing proteins localize to neural tissues, specifically giant fiber pathways and at putative sites of synaptic integration in the CNS and body wall musculature (Chapter III). Lan 3-2 positive proteins, especially MP66, are highly upregulated in the region of marked neural and regenerative plasticity, the segmental zone of giant fiber sensory field overlap and architomic fission (Chapter V). These observations place changes in MP66 expression (or posttranslational modification) at the exact time and place of neural morphallaxis during two distinction life history events of *Lumbriculus*, regeneration and reproduction. Until genetic, biochemical or pharmacological approaches (e.g., RNAi, antibody blockade, or morphallaxis-specific pathway inhibition) demonstrate the involvement of MP66 in the regulation of neural morphallaxis, its role will remain in a realm of correlation and circumstantial evidence.

Mass spectrometric analyses suggest the possible similarity between specific MP66 sequence fragments and conserved sequence masses are associated with the Ig domain of a receptor protein tyrosine phosphatase (RPTPs; Chapter V). This is particularly interesting since the Lan 3-2 epitope is a sugar-chain associated with two cell adhesion molecules in the leech CNS, Leech CAM and Tractin, and both of these proteins are members of the Ig superfamily of neural cell adhesion molecules (Huang et al., 1997; Jie et al., 1999; 2000). RPTPs and CAMs are known to play important roles during axon growth and guidance during neural development. In *Drosophila*, DPTP69D and DLAR have been implicated in both the guidance of motor axons (Desai et al., 1997; Krueger et al., 1996) and targeting of photoreceptor axons to specific synaptic fields (Clandinin et al., 2001; Maurel-Zaffran et al., 2001). Additionally, loss of function of

Dlar results in reduced synapse size and decreased synaptic terminal branch complexity (Kaufmann, et al., 2002; Broadie and Richmond, 2002). CAMs also play important roles during axon pathway formation and maintenance (Keynes and Cook, 1995; Tessier-Lavigne and Goodman, 1996). Moreover, increasing evidence suggests that neural CAMs also participate in activity-dependent plasticity during development, as well as during synaptic plasticity in adults (Fields and Itoh, 1996: Rutishauser and Landmesser, 1996). For example, posttranslational modifications of N-CAM govern spinal motor axon guidance (Tang et al., 1992) and Ap-CAM regulates synaptic facilitation during sensory-motor plasticity underlying behavioral sensitization in *Aplysia* (Zhu et al., 1995; Schacher et al., 2000). Both of these share extensive similarity to LeechCAM, which bears the Lan 3-2 glycoepitope. If future identification of MP66 verifies that this protein is a member of the Ig superfamily of neural cell adhesion molecules, it would represent an excellent candidate molecule for direct involvement in the synaptic plasticity that governs connectivity transformations associated with neurobehavioral morphallaxis.

Neural morphallaxis in *Lumbriculus variegatus* provides a tool for the further investigation of the cellular and molecular mechanisms of neural plasticity. Changes in giant fiber anatomy (i.e., diameter), biophysics (i.e., axonal conduction velocity), and integrative physiology (i.e., sensory fields,) which accompany neural morphallaxis in regenerating worm fragments (Drewes and Fourtner, 1990; Lesiuk and Drewes, 2001a; Chapter II), are likely mediated by cellular and molecular mechanisms shared with other types of neural plasticity, such as learning and memory. In these doctoral studies, I have

presented evidence that injury to the ventral nerve cord (VNC) of *Lumbriculus* may be necessary for the induction of regenerative processes (Chapter IV). For example, ectopic head formation only occurred following VNC ablation. This fact, together with results from other regeneration studies, implicates the damaged CNS as a source of cues that activate cellular plasticity events (Ambron and Walters, 1996; Shafer et al., 1998 Brecknell and Fawcett, 1996; Kumar et al., 2001; Fenrich and Gordon, 2004; Dinsmore and Mescher, 1998). Similarly, the cell signaling pathways underlying regenerative mechanisms in *Aplysia* are virtually identical to those involved in the cellular mechanisms of simple learning (Ambron and Walters, 1996; Moffett, 1996). It is therefore thought that molecular mechanisms underlying learning might have evolved from more primitive stress or injury responses of ancestral organisms (Walters, 1994). The demonstration here that injury of the CNS plays a role in segmental regeneration and asexual reproduction (Chapters III and IV) mechanistically links a wide range of biological processes: stress, injury and repair responses, asexual reproduction, and learning and memory. The adaptive values of each of these forms of plasticity are obvious, but the evidence that each might be evolutionarily related is not. However, a growing body of data supports the hypothesis that both asexual reproduction and simple forms of learning were derived from primitive injury responses (Ambron and Walters, 1996; Kandel, 2001). The result presented here that mechanisms of morphallaxis, typically linked to both asexual fission and segmental regeneration, can be dissociated from these general processes (Chapter II), suggests that each is a conglomerate of developmental processes recruited together to affect a particular life history event. These

data and interpretations support the idea that regenerative processes are a basic attribute of asexual fission in animals, rather than mechanisms of plasticity that evolved independently to mediate reproduction (Goss, 1969; Sanchez-Alvarado, 2000; Brockes et al., 2001).

The present studies of neural morphallaxis in *Lumbriculus* raises questions about axial body patterning, queries that have long intrigued developmental biologists. In this case, the question is how a quite small body fragment, which has lost both its head and tail ends, is able to determine its positional identity within the newly emerging animal. Determination of axial position and segmental polarity following transection is critical for the successful regeneration of missing body parts and the recovery of function. Studies indicate that axial patterning genes, utilized during early development, are reactivated following injury to coordinate the patterning of regenerating tissue. For example, Hox gene expression is induced during regeneration in planaria and in some echinoderms (Orii, et al., 1999; Thorndyke, et al., 2001). *Lumbriculus* provides a unique model for the study of positional specification during regeneration, since this annelid worm displays anterior-posterior gradients in neural anatomy and physiology that govern gradients in overt behavior, specifically rapid escape responses (Drewes and Fourtner, 1990). Thus, segments along the anterior-posterior body axis possess a specific identity based on their axial location and this identity can be monitored by electrophysiological and behavioral assays. The current studies have not investigated the role of axial patterning regulators in morphallaxis, however, these studies have produced the first detailed timeline of neural morphallaxis, in the context of two distinct life history events,

drawn from multiple kinds of data including behavioral, physiological, anatomical, and biochemical (Chapter II; Fig. 9). With the identification of anterior specific Hox genes in the polychaete worm, *Chaetopterus variopedatus* during embryonic development (Irvine and Martindale, 2000; 2001), it is likely that Hox genes also play a role during the respecification of regenerating tissues in worm fragments during morphallaxis. Moreover, β-catenin, an important activator of morphogenic signals (i.e., Wnts) in a number of developmental models, is expressed in segments just behind the head blastema early in segmental regeneration (Crawford, 2003). Future studies will determine the relationship between these early molecular signals and the process of axial patterning, with later events such as neural morphallaxis.

It is remarkable that isolated worm fragments, such as those amputated from posterior body regions, completely transform axial gradients in giant fiber sensory fields to match their new positional identity following regeneration (Drewes and Fourtner, 1990; Chapter II). In addition, in both segmentally regenerating and asexually reproducing worms, MP66 modifications are localized to axial regions undergoing morphallaxis (Chapters IV and V). Thus, an important outcome of the present studies is that behavioral, cellular, and molecular gradients (i.e., axial patterns) found in adult lumbriculid worms are uniquely plastic and therefore capable of remarkably rapid change as needed during regeneration associated with recovery from injury or during seasonal periods of asexual reproduction.

Most annelid worms regenerate tails from almost any segmental level, with the exception of leeches, which have limited regenerative capabilities (Berrill, 1952; von

Bernhardi and Muller, 1995). Among those oligochaete and polychaete worms that can regenerate tails, both the rate of segmental epimorphosis and quantity of segments regenerated vary among even closely related species (Berrill, 1952). Furthermore, most oligochaete worms have limited capacities for head regeneration. *Lumbriculus* is one of the exceptions. Over 70 years ago, Turner (1934) found that the rate of posterior regeneration (tail bud formation) occurs at a faster rate in fragments from more anterior body levels and this rate gradually decreases the more posterior the fragment origin. Thus, *Lumbriculus* exhibits a marked axial gradient in anterior regenerative capacity (Berrill, 1952). The present studies determined that *Lumbriculus* exhibits a greater capacity for ectopic head formation within a zone of enhanced capacity for plasticity (segments 48±10 in worms of 150 segments; Chapter IV), the exact body region comprised of segments that possess sensory inputs to both medial and lateral giant fiber systems, and consequently an overlapping area of activation for both head and tail behaviors (Chapter II). Remarkably, this zone of segmental plasticity is the preferred site for the formation of architomic fission planes during fragmentation by asexual reproduction (Chapters II and IV).

Gradients in regenerative capacity in this lumbriculid worm are reminiscent of morphogenic gradients reported in hydrozoan cnidarians, where head regeneration activity is highest in the apical end of the animal (Webster, 1966; Wolpert et al., 1972; Technau and Holstein, 1995). An organizer-like region in the hypostome, a small piece of tissue residing just below the tentacles in the apical end of *Hydra*, is capable of inducing a secondary body axis when grafted onto another polyp (Broun and Bode,

2002). Moreover, there is substantial evidence for the upregulation of genes encoding many players in the Wnt signaling pathway, including Wnt (HyWnt), Disheveled (HyDsh), GSK-3 (HyGSK3), and β-Catenin (Hyβ-Cat), within the hypostome (Hobmeyer, 2000; Holstein et al., 2003). The expression of these genes is also present in regenerating head tissue and, thus, is thought to be the molecular basis for the regeneration of axial gradients in *Hydra*. Drewes and Fourtner (1990) hypothesized that such an activating center may reside in both the head and tail ends of regenerating fragments of *Lumbriculus*. The observation that β-catenin expression is induced in the most anterior segments of a fragment following amputation (Crawford, 2003) suggests that this idea has merit. Based on the present demonstration that ectopic head formation is preferentially induced at a zone of segmental plasticity at the anterior-posterior behavioral interface, I predict that, as with late regeneration markers like MP66, early markers such as β-catenin would be preferentially induced here prior ectopic head formation or asexual fission. Future studies will confirm or refute this prediction. Thus, epimorphic segmental generation, whether injury-induce or intrinsically-driven, likely results from morphogenic influences of an activating or organizing center which forms near the anterior (head) blastema and the molecular features of this center may be especially deployed in this region of sensory field overlap/fission plane differentiation, a center of both neural and developmental plasticity.

In summary, these studies describe specific processes during neural morphallaxis that possess similarity to other forms of synaptic/neuronal plasticity. Neural morphallaxis is shown to be a transforming process that, although part of both repair and

reproductive events, can be recruited independently. Neural morphallaxis involves changes in the expression of proteins bearing specific glycoepitopes and these proteins are expressed in neural tissues at locations of morphallactic transformation. Moreover, specific segmental regions of the *Lumbriculus* body axis are particularly adapted for neural plasticity associated with recovery from injury or asexual fission. Although the exact mechanisms of neural morphallaxis remain to be determined, these studies provide a foundation for future investigations of the cellular and molecular mechanisms that govern neural morphallaxis in *Lumbriculus*.

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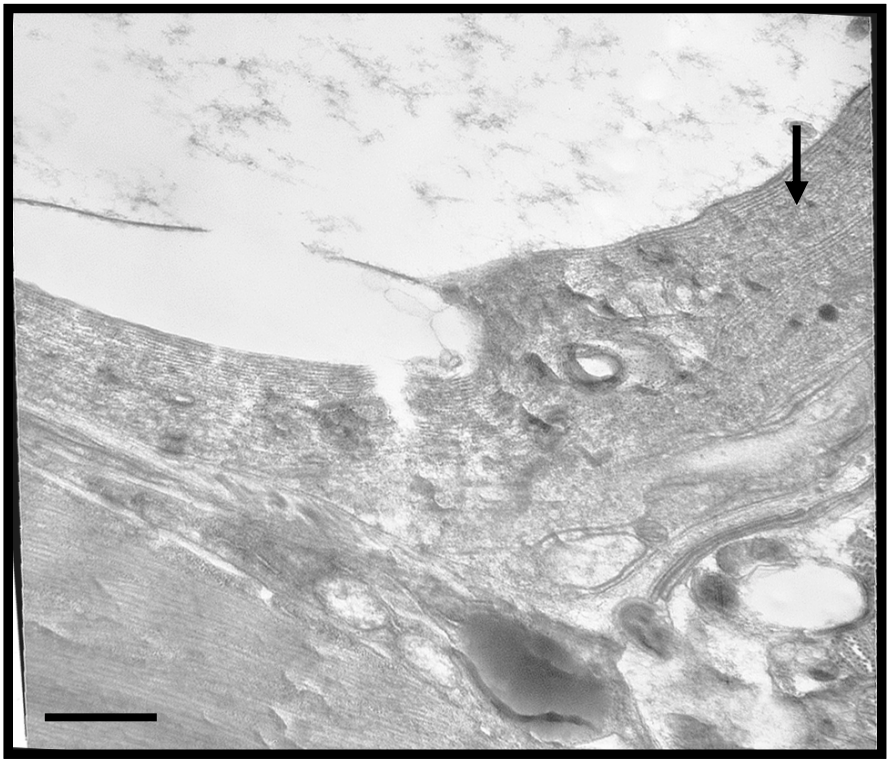
APPENDIX A

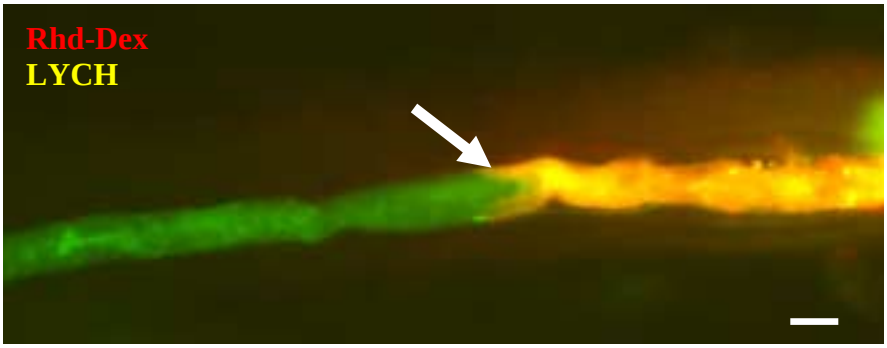
NATURE AND PLASTICITY OF INTERSEGMENTAL GIANT AXON CONNECTIVITY

A.1

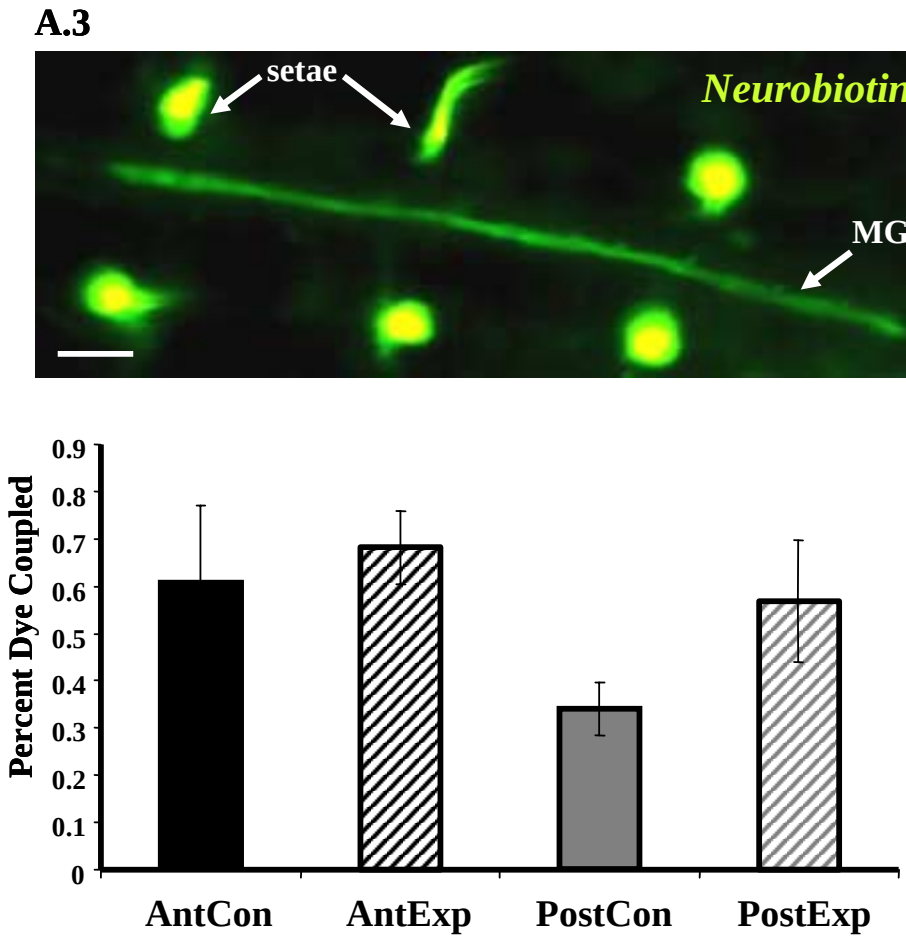


A.1- Lumbriculid giant fiber axons are myelinated. A transmission electron micrograph of a giant fiber (60,000X). Layers of myelin are visible surrounding the giant fiber membrane (arrow). Scale bar = 16nm.

A.2



A.2 – Septate Nature of the Giant Fiber Axons. A tracer dye mixture of lucifer yellow (LYCH) and rhodamine-dextran (Rhd-Dex) was injected into the medial giant fiber. Following an incubation period where the dye was allowed to fill numerous segments, the giant fiber/ventral nerve cord whole mounts were fixed, stained, and prepared for imaging. Rhodamine-dextran is a molecule of high molecular weight (10,000 MW) and thus did not pass through gap junctions found between the giant fiber axons. Standard fluorescence microscopy techniques were used to visualize the extent of dye passage. This representative image of the medial giant axon, illustrates that as in most oligochaete giant axons the pathways are, septate in nature (20X; arrow = septal boundary). The green LYCH staining in the adjacent axon indicates that these septate boundaries are sites of gap junctional communication (i.e., dye coupling) between giant interneurons. Areas of yellow fluorescence indicate segments where the dextran and lucifer yellow dyes overlapped in expression (i.e. sight of injection). Scale bar = 10μm.



A.3 - Previous studies have successfully demonstrated the use of a low molecular weight tracer, neurobiotin, as an indicator of electrical coupling between gap junctions (Vaney, 1991). Dye-coupling coefficients were calculated for anterior and posterior fragments prior to and following morphallaxis. Lucifer yellow or neurobiotin mixtures were injected into the medial giant fiber (MGF). This figure demonstrates that movement of dye from one cell to another (dye-coupling) occurs between giant fibers in adjacent body segments (Top). The fluorescent intensity of dye-coupled giant axonal segments was always less than the injected axonal segment because the presumptive gap

junctions in septal membranes limit the rate of diffusion between adjacent giant axons. Segmental boundaries can be deduced from the paired setae, which are autofluoresent and are segmentally arranged (one pair/segment). Scale bar = 30μm.

Anterior control (AntCon) fragments exhibited 60% dye coupling between medial giant fibers; where as posterior controls (PostCon) had 30% medial giant fiber dye coupling (Bottom). Dye-coupling coefficients demonstrated a trend toward an increase in medial giant fiber coupling in posterior regenerating fragments which were experiencing neural morphallaxis and acquiring a more anterior positional location along the body axis (n=5).

APPENDIX B

MASS SPECTROMETRY DATA OF UNKNOWN PROTEINS

Table 3 – Mass Spectrometry Data of Unidentified Protein Sequences

Protein	Accession No.	Sequence	M_rTh./Obs. (kDa)	XC>1
Juvenile Earthworm Library [Lr_JV2CF_27B04_SK plus]	CV072657			
Scan No.303		*R.RIVNNPSTELS LNFRSAHTR.T*	2311.21 /2312.22	2.11
Earthworm Fluorantene Exposed [Lr_PAHCF_27E04_M 13R]	CF799806			
Scan No.390		*K.YFKKQTEESA CK.I*	1460.70 /1461.70	2.11
Earthworm Head Enriched library [Lr_CHECF_26C04_M 13R]	CO378435			
Scan No.198		*R.FIDRAIDQWR R.W*	1474.78 /1475.78	2.05
Earthworm Head Enriched library [Lr_CHECF_20E04_M 13R]	CO378185			
Scan No.198		*K.LYNLGHSCTR LWGGGSVIQR.P*	2216.13 /2217.13	2.01
Eisenia andrei midgut cDNA library [BP524931]	BP524931			
Scan Nos. 463 - 466		*K.HFEFSQLEIY PQEISK.S*	1993.98 /1994.9864	2.13
Eisenia andrei midgut cDNA library [BP524419]	BP524419			
Scan Nos. 463 - 466		*K.VRADWHPTG PTWCPTDR.G*	1993.92 /1994.9295	2.08
Eisenia andrei midgut cDNA library [BP524728]	BP524728			
Scan Nos.463 - 466		*K.VRADWHPTG PTWCPTDR.G*	1993.92 /1994.9295	2.08

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Martinez VG, Reddy PK, and Zoran MJ (2005b). Asexual reproduction and segmental regeneration, but not morphallaxis, are inhibited by boric acid in *Lumbriculus variegatus* (Annelida: Clitellata: Lumbriculidae). Hydrobiologia (in press).

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