THE ROLE OF SODIUM INFLUX VIA VOLTAGE-GATED SODIUM CHANNELS
IN REGENERATION OF *LUMBRICULUS VARIEGATUS*

By

Manal Alkhathlan

A Thesis Presented to

The Faculty of Humboldt State University

In Partial Fulfillment of the Requirements for the Degree

Master of Science in Biology

Committee Membership

Dr. Bruce A. O’Gara, Major Professor, Committee Chair

Dr. Jacob P. Varkey, Committee Member

Dr. Edward Metz, Committee Member

Dr. Casey R. Lu, Committee Member

Dr. Michael R. Mesler, Graduate Coordinator

July 2015
Abstract

THE ROLE OF SODIUM INFLUX VIA VOLTAGE-GATED SODIUM CHANNELS
IN REGENERATION OF *LUMBRICULUS VARIEGATUS*

Manal Alkhathlan

The California Blackworm (*Lumbriculus variegatus*) has great ability to regenerate from small fragments into a new worm depending on two forms of regeneration: epimorphosis and morphallaxis. Both patterns of regeneration work in concert with each other in order to ensure the survival of the organism. Regeneration of the head segments following amputation produces 7-8 new segments; while the number of regenerated tail segments is dependent on the time following amputation. The nature, origin, and position of cells that are involved in formation of new tissues during regeneration in *L. variegatus* for both head and tail regeneration are not completely known. In this study I’m trying to find the nature, origin, and position of the neoblasts and investigate if the neoblasts that migrate into the cut site are cells derived from differentiated tissues already in the region of the wound by using EdU staining. The results showed that stem cells (neoblasts) are randomly scattered in the body and few in number. Upon injury these cells undergo hyperproliferation and migrate to the wound site for both head and tail regeneration. During head regeneration the results suggested a reprogramming of cells in the cut site upon injury due to the abundance of Na\(^+\) in cells in the injury site.
Recent studies showed that a change in the cell’s resting potential influences regeneration. In this study, I exposed the worms to the voltage-gated sodium channel blocker tricaine (TMS) and examined somatic regeneration of heads and tails following body transection. Regeneration of worms was examined for 10 days after amputation either in the presence of TMS or in control pond water. Regeneration of both new head and tail body segments was reduced in the presence of TMS, especially in the tail. Head regeneration in the presence of TMS showed defective morphology with no well defined segments. Both effects of sodium transport into the cells in the bud region were examined by CoroNa Green staining, and somatic cell regeneration in the bud by EdU. The number of dividing cells in the bud region for both head and tail TMS-treated worms decreased significantly, although number of dividing cells that were scattered around the remainder of the body didn’t show a significant change compared to control worms. The number of proliferating cells in the bud region showed a strong relationship with the concentration of Na⁺ in the cells in the bud region. To further investigate the effects of sodium influx on regeneration, monensin, a sodium ionophore, was used in TMS-treated worms. These worms did not show increased regeneration ability compared to TMS-treated worms.

In combination, these result suggest that sodium influx via voltage-gated sodium channels has effects on both head and tail regeneration that affects cell migration of dividing cells that are scattered around the body and dividing cells in the bud region.
Acknowledgments

I would like to thank a number of people for the support and guidance through my graduate studies. I would like to express my gratitude to my advisor Dr. Bruce O’Gara for his guidance, patience, and support, during my graduate education at Humboldt State University. Special thanks also to Dr. Jacob Varkey for his guidance, and encouragement during my graduate education and providing assistance in both knowledge, and in lab supplies for the entirety of the project. I also want to thank my graduate committee members, Dr. Casey Lu, and Dr. Edward Metz for being on the graduate panel and providing advice and guidance. Special thanks also to Dr. Sprowles for her guidance and support. Also I want to thanks Darrell Burlison for providing lab supplies.

I want to express my special gratitude to my country Saudi Arabia for providing me with this opportunity to study abroad and the financial assistance during my stay in the United States. Finally I am especially thankful for the love and support that I have received from my family not only through graduate school but also throughout life. I want to express my eternal gratitude to all members from my family especially my Father Abdullah Alkhathlan and my mother Sara Alessa and I hope I’ve made you proud. Your love has given me the confidence and freedom to achieve my dreams. For that, I cannot thank you enough. I love you more than words can express.
Table of Contents

Abstract ......................................................................................................................... ii
Acknowledgments .......................................................................................................... iv
Table of Contents ............................................................................................................. v
List of Figures ................................................................................................................ vi
Introduction .................................................................................................................. 1
  Regeneration in Annelids ............................................................................................... 3
  Voltage-Gated Sodium Channels .................................................................................. 5
  Tricaine Methanesulfonate ............................................................................................ 7
  Monensin ..................................................................................................................... 8
  Bioelectrical signals ..................................................................................................... 10
Methods and Materials .................................................................................................. 13
  Transections to Produce Regenerating Body Fragments .............................................. 13
  Tricaine Methanesulfonate (TMS) Treatments ............................................................. 15
  Monensin Treatments .................................................................................................. 15
  EdU Staining Protocol ................................................................................................. 16
  CoroNa Green Staining Protocol ................................................................................ 18
  Scanning Electron Microscopy .................................................................................... 19
  Transmission Electron Microscopy .............................................................................. 19
  Statistical analysis of regeneration ............................................................................. 20
Results ............................................................................................................................. 21
  Regenerating Body Fragments ..................................................................................... 21
  Effects of Tricaine Methanesulfonate on Regeneration ................................................ 23
  Effect of Monensin on Regeneration .......................................................................... 30
  Monensin Treatment Did Not Increase Regeneration in Worms Treated with TMS ... 35
  Stem Cell (Neoblast) Location, Division and Migration During Regeneration .... 37
  The Effects of Sodium Flux Inhibition on Cell Division During Regeneration ....... 48
  Sodium Distribution During Regeneration ................................................................... 54
  Sodium distribution during regeneration in TMS-treated worms ................................. 60
  Ultrastructure Level ..................................................................................................... 62
Discussion ....................................................................................................................... 66
  Regenerating Body Fragments ..................................................................................... 66
  Stem Cells (neoblast) location, division and migration during regeneration .......... 67
  Sodium Distribution During Head and Tail Regeneration ............................................. 69
  Effects of Tricaine Methanesulfonate on Regeneration ............................................... 70
  Monensin treatments .................................................................................................. 72
References ....................................................................................................................... 74
List of Figures

Figure 1. Regeneration in the oligochaete worm Lumbriculus variegatus..........................5
Figure 2. Mechanism of Sodium Transport by Monensin......................................................9
Figure 3. A Logical Schematic of One Possible Bioelectric Cascade During Regeneration..........................................................................................................................11
Figure 4. Transections to Produce Regenerating Body Fragments...........................................14
Figure 5. Lumbriculus variegatus Head and Tail Regeneration from Posterior and Anterior Fragments..................................................................................................................22
Figure 6. Head and Tail Regeneration Differences between Fragments from Anterior, Posterior, and Middle Parts of the Body..................................................................................24
Figure 7. Effects of Tricaine Methanesulfonate on Head Regeneration.................................25
Figure 8. Effects of Tricaine Methanesulfonate on Tail Regeneration.................................28
Figure 9: The Temporal Effects of TMS Treatment on Head and Tail Regeneration...........29
Figure 10. Effect of Monensin on Head and Tail Regeneration............................................31
Figure 11. Effect of Monensin and Na+ on Head and Tail Regeneration.............................33
Figure 12. Effect of Monensin and Na+ on Head and Tail Regeneration at 18 hr after transection ........................................................................................................................................34
Figure 13. Monensin Did Not Increase Regeneration in Worms Treated with TMS.........36
Figure 14. Cell Division During Regeneration and Neoblast Proliferation and Migration in Head Blastema..................................................................................................................................38
Figure 15. Cell Division During Regeneration and Neoblast Proliferation and Migration in Head body ..................................................................................................................................................40
Figure 16. Comparison of Cell Proliferation During Head Regeneration between Head Cut Site, Blastema and Non-Cut Site..........................................................................................41
Figure 17. Cell Division During Regeneration and Neoblast Proliferation and Migration in Tail Blastema. The left column shows Hoechst staining to indicate the number of cells in the image........................................................................................................................................43
Figure 18. Cell Division During Regeneration and Neoblast Proliferation and Migration in Tail body..................................................................................................................................................45
Figure 19. Comparison of Cell Proliferation During Tail Regeneration between Tail Cut Site, Blastema and Non-Cut Site..................................................................................................46
Figure 20. Cell Division During Regeneration and Neoblast Proliferation and Migration in Head and Tail bud from middle segments..................................................................................47
Figure 21. TMS Treatment Reduced the Number of Dividing Cells in the Regenerating Bud Region of the Head..................................................................................................................49
Figure 22. Comparison of the Number of Dividing Cells in the Regenerating Bud Region of the Head between TMS Treatment and Control...........................................................................50
Figure 23. TMS Treatment Reduced the Number of Dividing Cells in the Regenerating Bud Region of the Tail..................................................................................................................52
Figure 24. Comparison of the Number of Dividing Cells in the Regenerating Bud Region of the Tail between TMS Treatment and Control...........................................................................53
Figure 25. Sodium Distribution in Intact Worms......................................................................54
Figure 26. Distribution of Intracellular Sodium in Head Bud Region During Regeneration ..................................................................................................................................................56
Figure 27. Distribution of Intracellular Sodium in Tail Bud Region During Regeneration.. ........................................................................................................................................................................58
Figure 28. TMS Inhibits Sodium Flux in the Bud’s Cells During Head Regeneration..... 60
Figure 29. TMS Inhibits Sodium Flux in The Bud’s Cells During Tail Regeneration .... 61
Figure 30. Monensin increased Sodium Flux in The Bud’s Cells During Head and Tail in Early Stages of Regeneration.................................................................................................................................................62
Figure 31 : Scanning Electronic Micrograph........................................................................................................................................63
Figure 32. Transmission Electronic Micrograph Thin Section of Head Regeneration at Day Five........................................................................................................................................64
**Introduction**

*Lumbriculus variegatus* is a freshwater metameric worm, meaning that it is a segmented animal formed of repeated body parts (Zoran, 2010). These worms are also known as California blackworms, blackworms, or mudworms. The biological classification of this organism is as follows: Kingdom: Animalia, Phylum: Annelida, Class: Oligochaeta, Order: Lumbriculida, Family: Lumbriculidae, Genus: *Lumbriculus*, Species: *Lumbriculus variegatus*. The evolutionary relationships between these worms and other annelids are not completely deduced (Drewes, 2001).

*Lumbriculus variegatus* resides in shallow fresh water such as ponds, lakes, marshes, and swamps, where it feeds on microorganisms and algae (Brinkhurst and Gelder, 1991; Drewes, 2001). These worms are found widely in North America and Europe and can be easily collected during the spring and early summer months; where they are commonly found beneath layers of decomposing leaves or sediments near the base of emerging vegetation, such as cattails.

Adult animals are about 10 cm in length (approximately 200-250 segments) and 1.5 mm in diameter (Drewes, 2001). They use their heads to forage in sediments and debris, while their tails extend up into the water column for gas exchange (Drewes, 2001). Sexually mature individuals of *L. variegatus* arise at low frequencies in diploid as well as polyploid populations, and a great range of chromosome counts are reported: $2n = 34, 68, c. 85, 136, and c.185$; the highest number may represent 11- ploid individuals (Christensen, 1980). Genetic sequences for this worm are rare with very little information
published about the genomic DNA. However, more information about the mitochondrial DNA has been published (Gusafsson et al., 2008). Genetic variations between populations in different geographic locations indicate that what was assumed to be a single species of *L. variegatus* may in fact be three different species (Gusafsson et al., 2008). Although the taxonomic status of these worms is unclear, it appears that worms derived from the population used in this study will remain *Lumbriculus variegatus*.

During the summer months, sexual reproduction was recently documented, which as seen in many earthworms, involves copulation and sperm exchange generating transparent cocoons containing embryos surrounded by a jelly-like matrix. Each cocoon contains 4-11 fertilized eggs that undergo direct embryonic development with no larval stage. The cocoons are released by the adult worms into the environment and protect the embryos as they develop into worms (Drewes, 1990; Tweeten & Vang, 2011; Tweeten & Danielson, 2015).

Laboratory populations of *L. variegatus* are generally smaller than field collected worms with 4-6 cm in length (~ 150 segments) being a typical length for laboratory-raised worms, and they never reach sexual maturity or produce cocoons. Reproduction in the laboratory occurs by asexual fragmentation, which is a self-produced, mechanical breaking of the organism into two or more pieces, followed by regenerative growth of missing parts in each fragment (Drewes, 1996; Gustaffson et al., 2008). Each surviving fragment then undergoes rapid regeneration of body segments to form a new head end, tail end, or both ends. Eventually each fragment grows into a normal sized worm.
comprised of a combination of older and newer segments, representing two or more
generations of development. (Drewes, 1999).

The *Lumbriculus* body possesses anterior-posterior gradients in both anatomy and
physiology. The anterior 17 segments contain the brain, conical prostomium, mouth,
muscular pharynx, as well as male and female sex organs (the worms are
hermaphroditic), are darkly pigmented and wider than posterior segments. (Drewes,
2001; Jaimeson, 1981). Although more posterior segments appear similar to one another,
there are important differences in their structure and function along the body. For
example, the posteriormost 30 segments are unique in that they have respiratory
adaptations, and a large population of photoreceptor cells exists in the body wall
(Jamieson 1981; Drewes and Fourtner 1989).

*L. variegatus* has been used to examine a number of biological phenomena such
as: patterned regeneration of lost body parts, blood vessel pulsations, the swimming
reflex, peristaltic crawling behavior, giant nerve fiber action potentials, and sublethal
sensitivity to pharmacological agents or environmental toxicants (Drewes, 2001).

**Regeneration in Annelids**

Regeneration is a feature in some animals that gives them the ability to replace a
lost body parts after being damaged or lost. The capability of animals to regenerate varies
considerably depending on the body part considered and type of animal (Zoran, 2010).
For example, some annelids have a phenomenal capacity for regeneration. However, not
all annelids have the ability to regenerate lost segments and, of those who do, their ability
to regenerate head segments varies between species.

Regeneration in *L. variegatus* involves both cell migration and cell proliferation (Tweeten et al., 2008). Epimorphosis and morphallaxis are the two major forms of regeneration mechanisms in annelids. Epimorphosis is a pattern of regeneration that forms a blastema, which is a mass of undifferentiated cells capable of growth and differentiation (Yoshida-Noro et al., 2010). An example of epimorphosis occurs during regeneration of anterior or posterior segments. Such regeneration requires the formation of a bud, which contains the stem cells (neoblasts) that differentiate into new head or tail segments (Zoran, 2010). Morphallaxis, alternatively, is a pattern of regeneration that does not involve any differentiation of stem cells but involves the transformation of existing body tissues into newly organized structures with little new cell growth (Martinez et al., 2004). An example of morphallaxis, occurs when body segments in middle and posterior regions spectacularly transform their identity following transection into anterior or posterior segments. In other words, segments can transform their anatomy and physiology to take on the role of a segment from a very different region than the segment’s original location (Drewes, 2001).

The great ability of *L. variegatus* to regenerate from small fragments into a new worm depends on the two forms of regeneration: epimorphosis and morphallaxis (Drewes, 1996). Regeneration of the head segments following amputation produces 7-8 new segments; while the number of regenerated tail segments is dependent on the time following amputation (Martinez et al. 2004) (Figure 1).
Figure 1. Regeneration in the oligochaete worm Lumbriculus variegatus. The figure illustrates the two major forms of regeneration mechanisms in annelids: epimorphosis and morphallaxis. Epimorphosis is a pattern of regeneration that forms a blastema, which is a mass of undifferentiated cells capable of growth and differentiation. Morphallaxis, alternatively, is a pattern of regeneration that does not involve any differentiation of stem cells but involves the transformation of existing body tissues into newly organized structures with little new cell growth (Zoran, 2010).

**Voltage-Gated Sodium Channels**

Voltage-gated sodium channels are involved in the initiation and propagation of action potentials in cells that can be electrically excited such as nerve, neuroendocrine and muscle cells. Cells that cannot be electrically excited can also express voltage-gated sodium channels at low levels but the role of these channels in such cells is unclear (Rush et al. 2003; Kew et al. 2010). Voltage-gated calcium channels are believed to have given rise to voltage-gated sodium channels at the origin of the nervous system (Zakon et al.)
A gene similar to animal sodium channels was found in a single-celled choanoflagellate. Searching across several different animal genomes revealed that most lineages retained similar homologs. Some of these lineages have never possessed a nervous system, for example, the Placozoa (Liebeskind et al. 2011).

Sodium channels are made of a large α subunit that is associated with smaller β subunits. The core of the channel is made of the α subunit and can function on its own. The auxiliary β subunits are crucial for the kinetics and voltage sensitivity of the channel gating. As well, the β subunits are responsible for channel localization and interaction with the extracellular matrix, intracellular cytoskeleton and cell adhesion molecules. The α subunit is comprised of four homologous domains (I-IV), each containing α helices (S1-S6) across the membrane and a further pore loop flanked by the S5 and S6 segments (Catterall et al. 2003). Amino acid residues that are positively charged are found in the S4 segments at every third position. The depolarization of the cell interior causes the charged amino acids to move towards the outer surface of the membrane, which causes the channel to open. Domains III and IV are connected by a short intracellular loop that functions as the inactivation gate leading to a folded channel structure and the blockage of the pore from the inside when the membrane is depolarized for a sufficient amount of time (Kew et al. 2010; Tasi et al. 2013).

Voltage-gated sodium channels are activated and inactivated rapidly upon the depolarization of the cell membrane. When sodium ions enter the cell via the open channel pore, the membrane is depolarized, initiating the rising phase of the action potential (Frank et al. 2003).
Voltage-gated sodium channels exist in three states, activated (open), deactivated (closed) and inactivated (closed). Normally, the membrane is at its resting potential before the occurrence of the action potential and the sodium channels are functionally closed due to the closed state of the activation gates. Prior to activation of the channel, the inactivation gates are in an open state. The opening of the activation gates occurs as a result of depolarization, which induces movement of the positively-charged amino acids in the S4 segments, which in turn leads to opening of the pore and the flow of positively charged sodium ions into the cell via the open channel pore (Rush et al. 2007; Tasi et al. 2013). The neuronal membrane voltage is depolarized as a result. At the peak of the action potential, the inactivation gates close, which functionally closes the channel even though the activation gates remain open at this point in time. An inactivation gate can be supposed to be a sort of a plug embedded on domains III and IV of the intracellular α subunit of the channel. The flow of sodium ions through the channel is impeded when the inactivation gate closes; this terminates the depolarizing phase of the action potential. The channel is termed as inactivated when the inactivation gate is closed (Rush et al. 2007).

**Tricaine Methanesulfonate**

Tricaine methanesulfonate (TMS), also called MS-222, is a widely used anesthetic agent that is approved in some countries such as the United Kingdom, United States and Canada for provisional use (Carter et al. 2011). TMS and other anesthetic agents are commonly used to sedate fish and amphibians, especially when handling,
during transport or surgery (Mercy et al. 2013). Anesthesia acts by suppressing both the central and peripheral nervous systems, which reduces the animal’s sensory perceptions and voluntary movements (Carter et al. 2011).

While TMS is known to be a local anesthetic compound, it acts systemically following absorption via the skin and gills from anesthetic baths (Carter et al. 2011). In the body, TMS is rapidly metabolized through acetylation and excreted. Both TMS and non-polar metabolites are excreted through the gills of fish. However, unmetabolized TMS and other polar metabolites are passed to the kidney for excretion with the plasma half-life of TMS being estimated to be about 1.5-4 hours. Since the drug is rapidly eliminated, no detectable amounts of TMS are found in urine and whole blood (Carter et al. 2011). The previous information describes elimination of TMS from vertebrate animals; however, the pharmacokinetics and tissue distribution of TMS in *Lumbriculus* are unknown.

**Monensin**

Ionophores are compounds that transport ions as a lipid-soluble component across the phospholipid bilayer. Monensin is a member of a group of polyether ionophore antibiotics. Monensin is an antibiotic produced by *Streptomyces cinnamomensis*. Being an ionophore, monensin A functions in the transport of sodium cations across the phospholipid bilayer of biomembranes. Monensin A has been used as a nonhormonal growth promoter and coccidiodiostat growth promoter (Łowicki et al. 2013).
Monensin selectively binds sodium at an affinity ten times greater than its nearest biological competitor $K^+$ (Mollenhauer 1990). Monensin is composed of non-polar hydrocarbons that allow the sodium-monensin complex to be freely soluble in lipid membranes. Through this binding, sodium-monensin complex is capable of crossing the membrane. Once the complex reaches the interior membrane, it releases the sodium ion and binds a proton. It then returns through the membrane and exchanges the proton for another sodium ion at external surface (Mollenhauer 1990) (Figure 2).

Figure 2. Mechanism of Sodium Transport by Monensin (Łowicki et al., 2013).
Bioelectrical signals

In 2007, Michael Levin said in his review that regeneration is partly controlled by electrically-mediated signals. Supporting this hypothesis are three lines of evidence: (i) strong currents accompany regeneration events, and their magnitude, direction and spatial distribution is often different in the wounds of species that normally do not regenerate; (ii) inhibition of endogenous currents specifically abrogates regeneration; and most strikingly (iii) artificial induction of currents can induce a significant degree of regeneration in normally non-regenerating species.

Bioelectrical signals are able to guide the morphogenesis of structures on several scales (from single cells to whole embryonic axes). Thus, the linking of different levels of morphogenetic organization by ion flux-dependent mechanisms is an important component of systems biology (Figure 3); such mechanisms allow the coordination of subcellular polarization mechanisms with long-range (potentially organism-wide) morphogenetic polarity, and provide profoundly powerful and flexible system of cellular control.
Figure 3. A Logical Schematic of One Possible Bioelectric Cascade During Regeneration. The figure shows a possible bioelectric cascade from injury to the expression of specific transporters in the wound. These set up specific pH and membrane voltage domains, as well as long-range electric fields and epigenetic signals that ultimately affect the number, position and types of cells in the blastema. These changes
are coordinated with downstream genetic cascades to restore morphology of the tissue or organ being regenerated (Levin, 2007).

The first objective of this study is to examine the nature, origin, and position of cells that are involved in formation of new tissues during regeneration in *L. variegatus* for both head and tail regeneration and investigate if the neoblasts that migrate into the cut site are cells derived from differentiated tissues already in the region of the wound. To study this I will use EdU, which is a nucleoside analog of thymidine and is incorporated into DNA during active DNA synthesis. I will use it in intact worms (in vivo) during different stages of regeneration to observe cell proliferation and cell migration. This will help to discover the potential usefulness of *L. variegatus* as a model system in which to study the regulation and molecular processes of regeneration.

The second objective of this study is to determine if the influx of sodium through voltage-gated sodium channels is required for regeneration in *L. variegatus*. A recent study showed that the influx of sodium through voltage-gated sodium channels was essential to initiate regeneration in embryonic tadpoles (Tseng et al., 2010). In order to test this I will expose worms to the voltage-gated sodium channel blocker TMS and examine its effects on somatic regeneration of heads and tails following body transection at the molecular level.

Lastly, I will use the sodium ionophore monensin and observe somatic regeneration of heads and tails following body transection to determine if sodium flux via monensin can rescue regeneration in TMS-treated worms; thereby showing that sodium ion influx is essential for regeneration.
Methods and Materials

*Lumbriculus variegatus* were laboratory reared in aquaria containing aerated artificial pond water with pieces of paper towels to act as a substrate. The artificial pond water had the following composition: 1 mM NaCl; 13 µM KCl, 4 µM Ca (NO\(_3\))\(_2\)·4H\(_2\)O; 17 µM Mg (SO\(_4\))·7 H\(_2\)O; 71µM HEPES buffer; pH 7.0 ± 0.05. Worms were fed commercial fish food one to two times per week. The asexually reproducing cultures have been maintained in the laboratory and were derived from animals originally purchased from Aquatic Foods (Fresno, CA). Worms used in these experiments were randomly chosen and were lacking any obvious morphological defects.

Transsections to Produce Regenerating Body Fragments

Worms used in this study were between 130 and 150 segments. Worms were cut into three pieces with a scalpel at intersegmental boundaries to obtain experimental body fragments that consisted of approximately 30-35 segments from anterior and posterior body regions and approximately 70-80 segments from the middle region of the body (Figure 4). Following transection, worms were assigned into different treatment groups. Control worms were maintained individually in 24-well tissue culture dishes containing pond water. Animal survival and segmental regeneration was observed daily for 7 or 10 days. Worms from each well were transferred individually onto a microscope slide and new regenerating heads and tails was visualized and imaged by using an Olympus SZX16 WI light microscope and Olympus DP73 camera. Measurements of new head and tail
growth were measured by using CellSens 1.6 software. Segments were counted as true segments based on the presence of defined intersegmental boundaries.

Figure 4. Transections to Produce Regenerating Body Fragments. Whole animals of approximately 130-140 segments in length were cut at ~30-35 segments from the head, and at ~105-110 resulting in three regenerating body fragments (light gray): one from the anterior that will regenerate a new tail, one from the original posterior that will regenerate a 7-8 segment head, and one from the middle region of the body (approximately segments 30 – 105) that regenerates both a 7-8 segment head and variable lengths of new tail. Numbers indicates the segments numbers.
**Tricaine Methanesulfonate (TMS) Treatments**

To examine the effects of blocking voltage-gated sodium channels on regeneration, worms were treated with tricaine methanesulfonate (TMS). TMS is a widely used anesthetic agent and nervous system suppressant that works by inhibiting the entrance of sodium (Na\(^+\)) into the cells via voltage-gated sodium channels (Carter et al. 2011). A solution of 1700 µM TMS (Acros Organic, Pittsburgh, PA, USA) in pond water was prepared and the pH was adjusted to 7.0 ± 0.05. The concentration of TMS used was determined in preliminary experiments that examine the effects of different concentrations of TMS (1700 µM, 1000 µM, and 500 µM) to determine which concentration resulted in most profound blocking of regeneration. Immediately upon transection, 120 worms were maintained individually in pond water containing 1700 µM TMS for 7 days. Since TMS is light sensitive, the worms and solution were protected from light by wrapping the 24-well dishes in aluminum foil.

To further define the temporal effects of TMS treatment, 12 worms per different time interval were placed into pond water immediately following transection and then treated with TMS starting at 6 hr, 12 hr, 18 hr, 24 hr, or 48 hr post transection and continuing for the remainder of the experiment. Animal survival and segmental regeneration was observed daily for 7 days. Worms were imaged as above.

**Monensin Treatments**

A stock solution of 100 mM monensin (Acros Organic, Pittsburgh, PA, USA) was prepared in ethanol and kept in -20 °C. Following transection, 20 worms were maintained
individually in 24-well dishes containing 2.5 µM monensin with or without the addition of 2 mM of sodium gluconate (Santa Cruz Biotechnology, Dallas, TX) in pond water. Sodium gluconate was added to the pond water to increase the sodium concentration in the water, which normally has a low sodium concentration (1 mM). Animal survival and segmental regeneration was observed daily for 7 days.

To further examine the effects of monensin, 24 worms per time interval were placed into pond water for 6 hr, 18 hr, or 24 hr and then transferred into pond water contain 2.5 µM monensin for 45 minutes while protected from light. Some worms were also exposed to 2 mM sodium gluconate at the same time as monensin. Worms were then washed twice with pond water and transferred into 24-well dishes with each well containing control pond water. To determine if monensin could rescue worms from the effects of TMS, 24 worms were placed into 1700 µM TMS immediately following transection. TMS-treated worms were exposed to 2.5 µM monensin, and 2 mM sodium gluconate using the same protocol as used above for monensin alone. The concentration of monensin and sodium gluconate used showed in preliminary experiments that they had the best potentials in increasing regeneration.

**EdU Staining Protocol**

EdU staining was performed by following the manufacturer’s instructions for Click-iT® Plus EdU Imaging Kits (Life Technologies, Grand Island, NY) with the modifications described below. Regenerating worm body fragments were maintained individually in 24-well dishes. Three worms per day from each treatment were stained
with a solution consisting of either 500 µM EdU in pond water or 500 µM EdU in TMS pond water. Each worm was exposed to EdU by adding 250 µl of 500 µM EdU to each well. Following a 1.5 hr exposure time, EdU was removed and each well were fixed in 1 ml of 4% paraformaldehyde (Fisher Scientific, Pittsburgh, PA) overnight at 4°C. Fixative was then removed from each well and the worms in each well were washed twice for 15 minutes with 1 ml of 3% bovine serum albumin (BSA) (Fisher Scientific, Pittsburgh, PA) in PBS (Fisher Scientific, Pittsburgh, PA). The washing solution was removed and the worms in each well were permeabilized by the addition of 1 ml of 0.5 % Triton X-100 (Sigma- Aldehyde, St. Louis, MO) in PBS and incubated for 45 minutes at room temperature. The permeabilization buffer was removed from each well and the worms in each well were washed twice for 5 minutes with 3% BSA in PBS. Click-iT reaction cocktail (250 µL) consisting of 1x Click-iT reaction buffer, copper protectant, Alexa Fluor picolyl azide, and 1x reaction buffer additive was added to each well and were incubated at room temperature for 1 hour while protected from light. After 1 hour, the Click-iT reaction cocktail was removed and each well was washed once for 5 minutes with 1 ml 3% BSA in PBS and washed once with 1 mL of PBS for 5 minutes. To visualize nuclei, 250 µl of 1x Hoechst 33342 in PBS were added to each well and worms were incubated at room temperature for 1 hr protected from light. After 1 hr, each well was washed twice with PBS. Worms were imaged and analyzed using a fluorescence Zeiss microscope and AxionCam MRm camera using 488 nm excitation and 516 nm emission filters to visualize Alexa Fluor 488 dye and for Hoechst 33342, 350 nm
excitation and 461 nm emission filters were used. Cells numbers were counted by using ImageJ64 software.

**CoroNa Green Staining Protocol**

CoroNa Green staining was performed following the manufacturer’s instructions for CoroNa TM Green, AM *cell permeant (Life Technologies, Grand Island, NY) with the modifications described below. Stock solutions of 10 mM CoroNa Green were prepared in high-quality anhydrous DMSO. The 10 mM stock solution was diluted in pond water, TMS, or 2.5 μM monensin with 2 mM of sodium gluconate to obtain a concentration of 1 mM CoroNa Green. Regenerating worm body fragments were produced as describe above for all three different treatments and maintained individually in 24-well dishes. At 6, 12, 18, 24, 48, 72, 96, 120, 144, 168, and 192 hours post amputation 3 worms per different time interval from all different treatments were incubated in 1 ml of 1 mM CoroNa Green indicator dye solution in pond water (control), TMS, or 2.5 μM monensin with the addition of 2 mM of sodium gluconate. Worms were incubated in their respective solutions at room temperature while protected from light for 80 minutes. After 80 minutes, worms from each well were washed twice with pond water. Worms were immobilized by exposure to 3.7 % MgCl₂ for 3 minutes. Each worm was transferred individually onto a microscope slide and visualized under a fluorescence Zeiss microscope and AxionCam MRm camera using using 488 nm excitation and 516 nm emission filters.
**Scanning Electron Microscopy**

Both control and treated worms were flash frozen in liquid N\textsubscript{2} and freeze-dried in a Virtis Freezemobile overnight. Worms were mounted on stubs with TED Pella Mikrostik and sputter coated with gold. Then worms were imaged in an FEI Quanta 250 Scanning Electronic Microscope.

**Transmission Electron Microscopy**

Both control and treated worms (3 per different time interval) were fixed in 3% glutaraldehyde in 50 mM sodium cacodylate pH 7.0 buffer overnight at 4°C. Fixation of samples were performed by microwave tissue protocol; samples were chilled to 10 –15°C placed in the microwave for 40 seconds then cooled in an ice bath for 5 minutes then repeating the 40 seconds in microwave. Next, fixative was removed from the samples and 50 mM sodium cacodylate buffer was added and removed immediately and a second volume of fresh buffer was added and then placed in an ice bath for 5 minutes with this step being repeated again. The 50 mM sodium cacodylate buffer was removed and 1.5% osmium tetroxide was added and the specimen placed in the ice bath for 5 minutes followed by microwaving for 40 seconds and repeating this step again. The osmium tetroxide was removed and samples were washed with distilled water three times. A graded ethanol dehydration was then performed. The samples then were infiltrated with ERL 4221 resin to form blocks. The resin blocks were polymerized in a vacuum oven overnight. Then thin sections were cut using a glass knife and the sections were mounted
on copper grids. The grids were stained with uranyl acetate and lead citrate. Finally sections were imaged in a Philips EM208S TEM.

Statistical analysis of regeneration

Appropriate statistical tests were performed using SigmaPlot 12 software (Systat Software). Initially parametric ANOVAs were performed. If the data were not normally distributed or did not have equal variance (automatically checked by SigmaPlot), the appropriate nonparametric version of the statistic in question was used. The data are generally plotted or described as the mean ± the SEM.
Regenerating Body Fragments

Regeneration from posterior and anterior segments showed somewhat different growth patterns depending on whether a new head or tail was being regenerated (Figure 6A, B). Regenerating body fragments of both heads and tails were able to heal the wound completely within 24 hr of transection and initiate forming a blastema. By day 2, a blastema was completely formed and it continually elongated through day 3 with an average of 412.933 µm ± 19.248 (n= 24) growth in length for heads and an average of 494.992 µm ± 24.264 (n= 24) growth in the length of tails. Head regenerating body fragments initiated forming segments by day 4 and had formed 8 segments by day 6. Tail regenerating body fragments initiated forming segments by day 5 and by day 6, 10 segments were formed and the tail continued to grow (Figure 5A) (normal, unoperated animals also continually added segments to the tail). At day 7, 15 segments formed in the new tail and the length of the segments became longer. By day 8, approximately 20 segments were formed and a bud still continued to grow from the posterior terminus.
Figure 5. Lumbriculus variegatus Head and Tail Regeneration from Posterior and Anterior Fragments. A) Regeneration of head and tail fragments from posterior and anterior segments respectively, showing the initiation of the bud formation and completion of the formation of 8 head segments by day 6. In contrast, regenerating tails continued to grow and add new segments. Regenerating tissue is lighter in color. B) Regeneration of head and tail segments from posterior and anterior segments respectively, showed that head regeneration was complete by day 7, while tail regeneration continued until day 10 (and beyond).
Regeneration for head segments from the middle section of the transected worm showed similar results to head regeneration from posterior segments (Figure 6A), but tail regeneration from middle segments showed significant slower regeneration especially from day 5 to day 6. The two-way RM ANOVA indicated that there was a significant effect for body parts (p 0.012; F= 7.467; d.f. = 1, 105), days of regeneration (p<0.001; F= 144.330; d.f. = 6, 105), and interaction (p<0.001; F= 12.351; d.f. = 6, 105) (Figure 6B).

Effects of Tricaine Methanesulfonate on Regeneration

Over the 7-day observation period all middle and posterior sections survived 1700 µM TMS; however, only 80% of anterior sections survived (the sections that died showed inflammation on their head segments). Head and tail fragments exposed to 1700 µM TMS showed normal wound healing. Bud formation for heads was delayed until day 3 (compared to day two in untreated sections) while for tails; bud formation was delayed until day 4 (compared to day two in untreated sections).

Treatment of posterior and middle fragments with 1700 µM TMS for the duration of the 7-day assay period significantly reduced head regeneration when compared to controls. A two-way RM ANOVA was performed comparing the effects of TMS on regeneration over a period of seven days post transection. The two-way ANOVA indicated that there was a significant effect for drug treatment (p < 0.001; F = 18.948; d.f. = 1,105), days of regeneration (p < 0.001; F = 7.322; d.f. = 4, 105), and the interaction (p = 0.036; F = 2.671; d.f. = 4, 105) (Figure 7).
Figure 6. Head and Tail Regeneration Differences between Fragments from Anterior, Posterior, and Middle Parts of the Body. A) Comparison of head regeneration from the posterior end and from the middle of the body. Head regeneration from the middle section and head regeneration from the posterior end show no difference in growth. B)
Tail regeneration from the anterior section was significantly longer than the grown of tails from the middle section and showed significant difference by day 6.

Figure 7. Effects of Tricaine Methanesulfonate on Head Regeneration. A) Control head regeneration from posterior end sections at 7 days of regeneration showing 8 segments. B) Control head regeneration from middle sections at 7 days of regeneration showing 8 segments. C) Head regeneration from posterior end section for TMS-treated worm at 7 days of regeneration showing defective morphologic structure of the head regeneration and showing no well-defined segments and hook shape of the growing head. D) Head regeneration of a TMS-treated middle section at 7 days of regeneration showing no well-defined segments. E) Comparison of head regeneration between control untreated worms
and TMS-treated worms showed significant reduction of head regeneration in treated worms.

Head regeneration in TMS-treated fragments from the posterior end produced abnormal morphology, showed no well-defined segments and asymmetric growth sometimes resulting a hook-shaped regenerating head (Figure 7C). In contrast, head regeneration from the middle parts of the body also showed no well-defined segments but with less asymmetric growth (Figure 7E).

The impact of TMS was most prominent in regeneration of the tail, which was almost completely blocked in anterior fragments (Figure 8C), and had severe developmental defects in the number of segments produced. Treatment of posterior and middle fragments with 1700 µM tricaine methanesulfonate (TMS) for the duration of the 7-day assay period significantly reduced tail regeneration when compared to controls. A two-way RM ANOVA was performed comparing the effects of TMS on regeneration over a period of one to five days post transection. The two-way ANOVA indicated that there was a significant effect for drug treatment (p < 0.001; F = 610.728; d.f. = 1,105), days of regeneration (p < 0.001; F = 100.241; d.f. = 4, 105), and the interaction (p < 0.001; F = 73.625; d.f. = 4, 105) (Figure 8E).

To further define the temporal effects of TMS on regeneration, worms were treated with TMS at different times post transection. Regenerating heads exposed to TMS for the duration from 0 hr to 24 hr for head regeneration showed significantly less regeneration than controls. A one way ANOVA was performed comparing the effects of TMS on regeneration at day 7 days post transection. The one way ANOVA indicated that
there was a significant effect for drug treatment time \((p < 0.018; F = 3.824; \text{d.f.} = 6.51)\) (Figure 9). When exposure started 48 hours after transection, when blastema formation was complete, there was not a significant difference from controls \((p < 0.226)\) and even the defective morphological phenotype was not prominent in all head regenerates, but no well-defined segments were present (Figure 9). Regeneration of tails also showed the same results as regeneration of the head in that regeneration was significantly reduced from time zero up to 24 hr. A one way ANOVA was performed comparing the effects of TMS on regeneration at day 7 days post transection. The one way ANOVA indicated that there was a significant effect for drug treatment time \((p < 0.002; F = 6.373; \text{d.f.} = 6.51)\) (Figure 9), but when treatment was initiated at 48 hr, TMS did not significantly reduce regeneration and no well-defined segments was observed \((p < 0.168)\) (Figure 9) and also showed significant difference with worms exposed to TMS immediately post transection \((p < 0.001, n= 24)\). For both head and tail regeneration, there was a critical period immediately following transection where TMS reduced regeneration.
Figure 8. Effects of Tricaine Methanesulfonate on Tail Regeneration. A) Tail regeneration from control posterior end section at 7 days of regeneration showing ~ 19 segments. B) Tail regeneration from control middle section at 7 days of regeneration showing ~9 segments. C) Tail regeneration from posterior end sections for TMS-treated worm at 7 days of regeneration showing defective morphologic structure of tail regeneration with no well-defined segments. D) Tail regeneration from TMS-treated middle section at 7 days of regeneration showing no well-defined segments. E) Comparison of tail regeneration between control untreated worms from posterior end section and TMS treated worms from posterior end section show significant reduction of tail regeneration.
Figure 9: The Temporal Effects of TMS Treatment on Head and Tail Regeneration. A) Effects of TMS treatment on head regeneration when first applied at different intervals post transection. B) Effects of TMS treatment on tail regeneration when first applied at different intervals post transection.
Effect of Monensin on Regeneration

Monensin, which facilitates the transport of sodium cations across the phospholipid bilayer of biomembranes, was used to examine the effects of sodium influx on regeneration. Worms treated with different concentrations of monensin alone from immediately following transection through 7 days post transection showed significant concentration-dependent reductions in regeneration for heads. A two-way RM ANOVA was performed comparing the effects of different monensin concentrations over a period of one to seven days post transection. The two-way RM ANOVA indicated that there was a significant effect for drug treatment (p<0.001; F= 11.055; d.f.= 3, 88), days of regeneration (p<0.001; F= 311.370; d.f.= 2, 88), and the interaction (p<0.001; F= 5.555; d.f.= 6, 88). For tails a two-way RM ANOVA was performed comparing the effects of different monensin concentrations over a period of one to seven days post transection. The two-way RM ANOVA indicated that there was a significant effect for drug treatment (p = 0.002; F=5.920; d.f.= 3, 88), days of regeneration (p<0.001; F= 245.535; d.f.= 2, 88), and the interaction (p<0.001; F= 4.671; d.f.= 6, 88). Unlike treatment with TMS, monensin-treated worms were able to regenerate well-define segments and a normal head and tail morphology (Figure 10).
Figure 10. Effect of Monensin on Head and Tail Regeneration. All regenerating sections are from posterior and anterior ends to respectively regenerate a new head or tail.

A) Comparison of head regeneration between control and monensin-treated worms showed significant reduction in regeneration between control and 3 uM monensin.

B) Comparison of tail regeneration between control and monensin-treated worms showed significant reduction in regeneration between control and 3 uM monensin.
The addition of 1 mM of sodium gluconate along with monensin did not ameliorate the significant reduction in regeneration. A one-way ANOVA was performed comparing the effects of different monensin concentrations day days 3 post transection. The one-way ANOVA indicated that there was a significant effect for drug treatment in head regeneration (p<0.001; F= 35.274; d.f.= 3, 11) and for tail regeneration (p<0.001; F= 129.355; d.f.= 3, n= 11). When sodium was combined with 3 µM monensin, all worm sections died (Figure 11).

To examine if there is a critical period for monensin exposure and to attempt to eliminate the toxic effects of continuous exposure to monensin, worms were treated with 2.5 µM monensin with the addition of 1 mM of sodium gluconate at either 6 hr or 18 hr after transection for only 45 minutes. Treatments of worms at 6 hr showed similar results for worms treated immediately post transection as describe above (Figure 11). When 2.5 µM monensin with the addition of 1 mM of sodium gluconate was added to worms for only 45 minutes at 18 post transection head regeneration did not show an increase in growth compared to worms not treated with monensin. A two-way RM ANOVA was performed comparing the effects of monensin over a period of two to four days post transection. The two-way RM ANOVA indicated that there was not a significant effect for drug treatment (p= 0.072; F= 3.603; d.f.= 1, 42); however, days of regeneration (p<0.001; F= 206.753; d.f.= 2, 42), was significantly different, but not the interaction (p 0.936; F= 0.0663; d.f.= 2, 42). The statistical analysis for tail regeneration in that drug treatment was not significant (p= 0.066; F= 3.770; d.f.= 1, 42), days of regeneration was
significantly different (p<0.001; F= 62.003; d.f.= 2, 42), but not interaction (p 0.103; F= 2.403; d.f.= 2, 42) (Figure 12).

Figure 11. Effect of Monensin and Na+ on Head and Tail Regeneration. All regenerating sections are from posterior and anterior ends to respectively regenerate a new head or tail.

A) Comparison of head regeneration between control and monensin-treated worms with the addition of 1 mM Na+ showed significant reduction in regeneration between control and 3 uM monensin, and 2.5 uM.

B) Comparison of tail regeneration between control and monensin-treated worms with the addition of 1 mM Na+ showed significant reduction in regeneration between control and 3 uM monensin, and 2.5 uM. Even with the addition of sodium, monensin still reduced the amount of regeneration for the head and tail.
Figure 12. Effect of Monensin and Na+ on Head and Tail Regeneration at 18 hr after transection. All regenerating sections are from posterior and anterior ends to respectively regenerate a new head or tail. Comparison of head and tail regeneration between control worms and worms treated with monensin at 18 hr following transection for 45 minutes. Under these conditions, monensin treatment produced an increase of head regeneration bud (A), or tail regeneration (B).
Monensin Treatment Did Not Increase Regeneration in Worms Treated with TMS

To examine if monensin treatment could rescue worms treated with TMS, monensin was added to TMS-treated worms at 18 hr following transection for 45 min. The results did not show an increase in the length of the regeneration bud produced by monensin treatment for both head and tail regeneration. When 2.5 µM monensin with the addition of 1 mM of sodium gluconate was added to TMS-treated worms for only 45 minutes at 18 post transection head regeneration showed no significant increase a two-way RM ANOVA was performed comparing the effects of monensin over a period of two to four days post transection. The two-way RM ANOVA for head regeneration indicated that there was not a significant effect for drug treatment (p = 0.399; F = 0.755; d.f. = 1, 44), days of regeneration (p < 0.001; F = 17.596; d.f. = 2, 44), and the interaction (p = 0.735; F = 0.309; d.f. = 2, 42) and the results for tail regeneration for drug treatment (p = 0.566; F = 0.341; d.f. = 1, 38), days of regeneration (p < 0.001; F = 25.353; d.f. = 2, 38), and the interaction (p = 0.011; F = 5.195; d.f. = 2, 38) (Figure 13).
Figure 13. Monensin Did Not Increase Regeneration in Worms Treated with TMS. All regenerating sections are from posterior and anterior ends to respectively regenerate a new head or tail. A) Comparison of head and tail regeneration at 72 hr following transaction between TMS treated worms and worms treated with monensin at 18 hr post transection for 45 minutes. For both head and tail regeneration, monensin treatment did not produce an increase in the length of the regeneration bud.
Stem Cell (Neoblast) Location, Division and Migration During Regeneration

Worms were stained with EdU (5-ethynyl-2′-deoxyuridine), which is a nucleoside analog of thymidine and is incorporated into DNA during active DNA synthesis. EdU staining is indicative of cells that underwent cell division during the EdU incubation period. Staining at 6 hr, 12 hr, 18 hr, 24 hr, and 48 hr following transection was used to determine when and where the cell divisions were occurring during regeneration.

At 6 hr into regeneration of the head there were approximately 20 cells proliferating in the cut site (Figure 14A) and in other regions of the body dividing cells were randomly scattered and were approximately 155 (Figure 15A). By 12 hr post transection, the number of dividing cells in the head cut site increased to approximately 25 cells (Figure 14B) while number of proliferating cells in other parts of the body increased to approximately 350 cells (Figure 15B). At 18 hr post transection, dividing cells in the head cut site increased to approximately 35 cells (Figure 14C) and in other part of the body approximately 320 cells (Figure 15C). At 24 hr the initiation of blastema formation was clear by the accumulation of proliferating cells at the cut site and the number of proliferating cells in the head increased to approximately 45 cells (Figure 14D) and the number of dividing cells in the other parts of the body start to decrease to approximately 144 cells (Figure 15D). The number of proliferating cells dramatically increased to approximately 200 cells in the head blastema by 48 hr (Figure 14E), and in other parts of the body decreased into 50 cells (Figure 15E). Overall the results showed
that with time proliferating cells scattered around the body decrease, while cells in the cut site, around it and in the blastema increase (Figure 16).

Figure 14. Cell Division During Regeneration and Neoblast Proliferation and Migration in Head Blastema. The left column shows Hoechst staining to indicate the number of cells in the image. The middle column shows EdU staining, which indicates cells that have recently undergone cell division. The right column images are merged images of the
two images to the left of the merged image. Images show head regeneration from 6 hr to 48 hr post transaction for fragments from the posterior end.

A) At 6 hr post transection, there were ~20 proliferating cells around the cut site.  
B) By 12 hr the number of dividing cells in the cut site increased to ~25.  
C) By 18 hr following transection dividing cells increased to ~35.  
D) Initiation of blastema formation was clear by 24 hr and the number of proliferating cells in the bud region increased to 45 cells. 
E) By 48 hr post transection, the blastema was well formed and the number of cells increased to ~20. Long bright fluorescent structures in the EdU photographs that often occur at regular intervals in the images are autofluorescent setae.
Figure 15. Cell Division During Regeneration and Neoblast Proliferation and Migration in Head body. The left column shows Hoechst staining to indicate the number of cells in the image. The middle column shows EdU staining, which indicates cells that have recently undergone cell division. The right column images are merged images of the two images to the left of the merged image. Images show head regeneration from 6 hr to 48 hr post transection for fragments from the posterior end. All images show the body parts of the same worms as figure 15 and showing the same time intervals.

A) At 6 hr post transection, dividing cells were randomly scattered and were approximately 155.
B) By 12 hr the number of dividing cells increased to 350.
C) By 18 hr following transection, dividing cells increased to 320.
D) By 24 hr when the blastema was initiate formed, the number of dividing cells decreased to 144 cells.
E) By 48 hr post transection, the blastema was well formed and the numbers of cells decreased to 50 cells. Long bright fluorescent structures in the Edu photographs that often occur at regular intervals in the images are autofluorescent setae.

Figure 16. Comparison of Cell Proliferation During Head Regeneration between Head Cut Site, Blastema and Non-Cut Site. Analysis of cells proliferation between the head cut site, blastemal and around the cut site, and other parts of the body starting from 6 hr into regeneration until 48 hr when the blastema was completely formed. Cells proliferation in the cut site and around it gradually increased, while cells proliferating in other parts of the body gradually decreased.

At 6 hr into regeneration of the tail there were fewer dividing cells in the cut site compared to head cut site approximately only 3 cells (Figure 17A) and in other regions of the body dividing cells were randomly scattered and numbered approximately 90 cells (Figure 18A). Proliferating cells in the cut site by 12 hr post transection, increased to approximately 9 cells (Figure 17B) while number of proliferating cells in other part of the
body increased to approximately 111 cells (Figure 18B). At 18 hr post transection, dividing cells in the tail cut site increased to approximately 14 cells (Figure 17C) and in other part of the body to approximately 155 cells (Figure 18C). At 24 hr the initiation of blastema formation was clear by the accumulation of proliferating cells at the cut site and the number of proliferating cells increased to approximately 31 cells (Figure 17D) and number of dividing cells in the other parts of the body increase dramatically to approximately 400 cells (Figure 18D). The number of proliferating cells dramatically increased to approximately 127 cells in the tail blastema by 48 hr (Figure 17E), and in other parts of the body decreased to 189 cells (Figure 18E). Overall the results showed that with time, proliferating cells scattered around the body decreased in number, while cells in the cut site, around it and in the blastema increase (Figure 19).
Figure 17. Cell Division During Regeneration and Neoblast Proliferation and Migration in Tail Blastema. The left column shows Hoechst staining to indicate the number of cells in the image. The middle column shows EdU staining, which indicates cells that have recently undergone cell division. The right column images are merged images of the two images to the left of the merged image. Images show tail regeneration from 6 hr to 48 hr post transection for fragments from the anterior end.

A) At 6 hr post transection, there were ~ 3 cells proliferating around the cut site.
B) By 12 hr, the number of dividing cells in the cut site increased to ~9.
C) By 18 hr post transection the number of dividing cells increased to ~14.
D) Initiation of bud formation was clear by 24 hr and the number of proliferating cells in
   the bud region increased to 31 cells.
E) By 48 hr the blastema had formed and cells numbers increased to ~127. Long bright
   fluorescent structures in the EdU photographs that often occur at regular intervals in the
   images are autofluorescent setae.
Figure 18. Cell Division During Regeneration and Neoblast Proliferation and Migration in Tail body. The left column shows Hoechst staining to indicate the number of cells in the image. The middle column shows EdU staining, which indicates cells that have recently undergone cell division. The right column images are merged images of the two images to the left of the merged image. Images show tail regeneration from 6 hr to 48 hr post transection for fragments from the posterior end. All images show the body parts of the same worms as figure 18 and showing the same time interval.

A) At 6 hr post transection, dividing cells were randomly scattered and were approximately 90 in number. B) By 12 hr the number of dividing cells increased to 111. C) By 18 hr following transection, dividing cells increased to 115. D) By 24 hr when blastema had
formed, the number of dividing cells increased to 400 cells. E) By 48 hr post transection, the blastema was well formed and the numbers of cells decreased to 189 cells. Long bright fluorescent structures in the EdU photographs that often occur at regular intervals in the images are autofluorescent setae.

Figure 19. Comparison of Cell Proliferation During Tail Regeneration between Tail Cut Site, Blastema and Non-Cut Site. Analysis of cell proliferation between tail cut site, blastema and around the cut site, and other parts of the body starting from 6 hr into regeneration until 48 hr when the blastema was completely formed. Cells proliferation in the cut site and around it gradually increased, while cells proliferating in other parts of the body gradually decreased.

To further investigate the difference between head and tail regeneration, middle body fragments that regenerate both head and tail segments at the same time (c.f., Figure 4) were stained with EdU. Results showed that at 24 hr of regeneration hyperproliferation was observed in the whole body and cells were moving toward both
head and tail cut sites resulting in initiation of blastema formation (Figure 20A, H). By 48 hr into regeneration the head blastema was completely formed, but the tail blastema was not completely formed and cells migration continued (Figure 20B, I). By 144 hr into regeneration, head regeneration was complete with 8 segments, and tail regeneration had formed only 4 segments (Figure 20F, M).

<table>
<thead>
<tr>
<th>24 hr</th>
<th>48 hr</th>
<th>72 hr</th>
<th>96 hr</th>
<th>120 hr</th>
<th>144 hr</th>
<th>168 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
<td>E</td>
<td>F</td>
<td>G</td>
</tr>
</tbody>
</table>

**Figure 20.** Cell Division During Regeneration and Neoblast Proliferation and Migration in Head and Tail bud from middle segments. In the groups of images for head or tail regeneration, the left column of each panel shows Hoechst staining to indicate the number of cells in the image. The middle column of each panel shows EdU staining, which indicates cells that have recently undergone cell division. The right column of each panel merges the two images just to the left of the merged image. A-G) Images of head.
regeneration from day 1 to day 7 showed a high number of proliferating cells. H-N) images of tail regeneration showed high numbers of proliferating cells. Long bright fluorescent structures in the EdU photographs that often occur at regular intervals in the images are autofluorescent setae.

The Effects of Sodium Flux Inhibition on Cell Division During Regeneration

To understand the cellular basis for the reduction in regenerative caused by the blockage of voltage-gated sodium channels, EdU Imaging Kits were used to quantify the number and distribution of proliferating cells in regenerating buds. Cell proliferation in TMS-treated animals and control untreated animals was examined and compared. Although the number of dividing cells that were scattered around the body (not near the cut site) in TMS-treated animals were not significantly different than untreated animals through 7 days of regeneration, cells proliferation in sodium flux-inhibited animals was significantly decreased in the cut site and bud region compared to control.

Starting from 24 hr into regeneration, proliferating cells in the control head bud region continued to gradually increase until 8 segments were formed and by day 6, proliferating cells in the head bud region started to gradually reduce in number (Figure 21 A-G). TMS-treated worms showed no cell division in the head regeneration cut site region until day 3 when low numbers of dividing cells were observed (approximately 5 cells). The number of proliferating cells in the bud region increased by day 7 of regeneration to approximately 150 (Figure 21H-N). A two-way ANOVA was performed to compare the number of cells proliferating in the regenerating bud between control and sodium flux-inhibited animals through 7 days of head regeneration. The two-way
ANOVA indicated that there was a significant effect for drug treatment ($p < 0.001$; $F = 117.045$; d.f. = 1,14), days of regeneration ($p < 0.001$; $F = 20.858$; d.f. = 6, 14), and the interaction ($p = < 0.001$; $F = 29.558$; d.f. = 6, 14) (Figure 22).

Figure 21. TMS Treatment Reduced the Number of Dividing Cells in the Regenerating Bud Region of the Head. In the groups of images for control or TMS-treated worms, the left column of each panel shows Hoechst staining to indicate the number of cells in the image. The middle column of each panel shows EdU staining, which indicates cells that have recently undergone cell division. The right column of each panel merges the two images just to the left of the merged image. A-G) Images of control head regeneration from day 1 to day 7 showed a high number of proliferating cells while (H-N) images of head regeneration in tricaine-treated worms showed low numbers of proliferating cells.
Long bright fluorescent structures in the EdU photographs that often occur at regular intervals in the images are autofluorescent setae.

Figure 22. Comparison of the Number of Dividing Cells in the Regenerating Bud Region of the Head between TMS Treatment and Control. The number of dividing cells in the regenerating bud were observed and counted through 7 days of regeneration for both control and TMS-treated worms. A two-way ANOVA was performed to compare the number of cells proliferating in the regenerating bud between control and sodium flux-inhibited animals through 7 days of head regeneration. The two-way ANOVA indicated that there was a significant effect for drug treatment ($p < 0.001; F = 117.045; \text{d.f.} = 1,14$), days of regeneration ($p < 0.001; F = 20.858; \text{d.f.} = 6, 14$), and the interaction ($p < 0.001; F = 29.558; \text{d.f.} = 6, 14$).
Through 7 days of regeneration, the number of proliferating cells in regenerating tail buds continued to increase gradually and reached approximately 1312 cells by day 7 (Figure 23A-G). Tail regeneration in TMS-treated worms showed no cell division in the regenerating bud region until day 3 with low numbers of dividing cells were observed (approximately 15). The number of proliferating cells in the bud region increased by day 7 of regeneration to approximately 250 (Figure 23H-N). A two-way ANOVA was performed to compare the number of cells proliferating in the bud regeneration between control and sodium flux-inhibited animals through 7 days of tail regeneration. The two-way ANOVA indicated that there was a significant effect for drug treatment ($p < 0.001; F = 569.597; \text{d.f.} = 1,14$), days of regeneration ($p < 0.001; F = 115.514; \text{d.f.} = 6, 14$), and the interaction ($p = < 0.001; F = 60.859; \text{d.f.} = 6, 14$) (Figure 24).
Figure 23. TMS Treatment Reduced the Number of Dividing Cells in the Regenerating Bud Region of the Tail. In the groups of images for control or TMS-treated worms, the left column of each panel shows Hoechst staining to indicate the number of cells in the image. The middle column of each panel shows EdU staining, which indicates cells that have recently undergone cell division. The right column of each panel merges the two images just to the left of the merged image. Images of tail regeneration in control worms showed a higher number of proliferating cells and regeneration through day 9. Images of tail regeneration in tricaine-treated worms showed fewer proliferating cells.
Figure 24. Comparison of the Number of Dividing Cells in the Regenerating Bud Region of the Tail between TMS Treatment and Control. The number of dividing cells in the regenerating bud was observed and counted through 7 days of regeneration for both control and TMS-treated worms. A two-way ANOVA was performed to compare the number of cells proliferating in bud the regeneration between control and sodium flux-inhibited animals through 7 days of tail regeneration. The two-way ANOVA indicated that there was a significant effect for drug treatment (p < 0.001; F = 569.597; d.f. = 1, 14), days of regeneration (p < 0.001; F = 115.514; d.f. = 6, 14), and the interaction (p = < 0.001; F = 60.859; d.f. = 6, 14).
Sodium Distribution During Regeneration

Intracellular sodium concentrations were visualized using CoroNa Green, a fluorescent sodium indicator dye that exhibits an increase in fluorescence upon binding Na\(^+\) ions. In intact worms, the CoroNa Green signal varied between different parts of the body, with the signal being strong in the middle segments and was more modest in the head segments and very low in the tail segments (Figure 25 A-C).

Figure 25. Sodium Distribution in Intact Worms. A) The intact head shows a small number of positive cells for CoroNa signals (arrows). B) Segments in the middle of the body show a higher number of positive cells for CoroNa signals. C) The intact tail shows very few positive cells for CoroNa green signals. The large repeating fluorescent structures (especially prominent in [B]) are autofluorescent setae.
At 24, 48, 72, 96, 120, 144, 168 hours following transection, worms showed high number of cells positive for CoroNa Green signal in the regenerating head bud region but not in the areas of the body that were not undergoing regeneration, suggesting a significant increase in sodium concentration in the cells of the bud during regeneration (Figure 26). The CoroNa Green signal gradually reduced over time in the regenerating head buds while the buds continued to grow, and the CoroNa signal was almost abolished by 168 hr when the worms had formed the complete 8 segments normally found in head regeneration (Figure 26A-G).

Head regeneration in worms cut at different sites along the anterior-posterior axis of the body showed different concentrations of sodium in the regenerating bud. Worms cut at the middle segments as described above showed higher numbers of positive cells with the CoroNa green signal in the bud regeneration region through the 7 days of regeneration (Figure 26 H-N).
Figure 26. Distribution of Intracellular Sodium in Head Bud Region During Regeneration. The left column of each panel show cells positive for CoroNa Green, which binds to sodium ions. The right column of each panel merges the left column with normal light illumination image of the worm. The images compare the concentration of sodium in head regeneration from posterior fragments, and middle fragment through 7 days of regeneration. Head regeneration from posterior fragments showed its highest
concentration of sodium from day 3-5 days of regeneration (C, D, E). Sodium concentration gradually started to reduce starting at 144 hr post transection (F, G). Head regeneration from middle body fragments showed high concentrations of sodium from day 1 to day 6 (H-M). By day 7 the concentration of sodium was reduced (N).

Similarly to the head, regenerating tails showed higher numbers of cells positive for the CoroNa Green signal in the regenerating bud region but not in the rest of the body at 24, 48, 72, 96, 120, 144, 168 hours after transection (Figure 27). The number of cells positive for CoroNa Green signal in the head bud was higher than in the tail bud. The CoroNa Green signal gradually diminished over time in the regenerating tail bud while worms continued to grow. The signals continued to be present at only the tip of the growing tail, which is the only part of that region that was still regenerating (Figure 27G). Unlike head regeneration from middle segments, tail regeneration from middle segments showed lower positive signals of CoroNa Green fluorescence than when the transection occurred nearer the worm’s original rear end (Figure 27 H-N).
Figure 27. Distribution of Intracellular Sodium in Tail Bud Region During Regeneration. The left column of each panel shows cells positive for CoroNa Green, which binds to sodium ions. The right column of each panel merges the left column with normal light illumination image of the worm. Comparing the concentration of sodium in tail regeneration from anterior fragments, and middle fragment through 7 days of regeneration. Tail regeneration from anterior fragments showed its highest concentration
of sodium from day 2-5 (B, C, D). The sodium concentration gradually started to diminish on subsequent days and ended up accumulating only at the tip of the bud (E, F, G). Tail regeneration from middle body fragments showed the highest concentration of sodium at day 5-6 (L-M). By day 7, the concentration of sodium was reduced (N). Cells positive for CoroNa in the bud of tail from middle body fragments were fewer than in the bud of the tail from anterior fragments.
Sodium distribution during regeneration in TMS-treated worms

CoroNa Green fluorescence was used to demonstrate the effect of TMS in blocking voltage-gated sodium channels and inhibiting sodium flux. Worms treated with TMS immediately following transection showed significantly fewer cells positive for the CoroNa Green signal. Figure 29 showed the CoroNa Green signal in the regenerating head bud in TMS-treated worms for head regeneration bud at 24, 48, 72, 96, 120, 144 hours after amputation. At 24 and 48 hours of head regeneration, TMS treatment abolished the CoroNa Green signal. However, at later time points the CoroNa Green signal gradually increased in the regenerating head bud while worms continued growing even though TMS was still present. TMS treatment produced abnormal morphology in the regenerated head. Cells positive for CoroNa Green accumulates at one side of the regenerating bud correlating with the shape that forming in the head (Figure 28). It is notable that the CoroNa Green cells were present on the side showing the most growth.

Figure 28. TMS Inhibits Sodium Flux in the Bud’s Cells During Head Regeneration. The first row shows cells positive for CoroNa Green. The bottom row merges the top row image with the normal light illumination image of the worm. A-F) Images show the distribution of intracellular sodium in head bud region during regeneration. The repeating bright fluorescent structures are autofluorescent setae.
At 24, 48, and 72 hours following transection, regenerating tails treated with TMS immediately following transection showed no CoroNa Green signal and the bud did not grow. TMS-treated tails at 96, 120, and 144 hours following transection showed significantly fewer numbers of cells positive for the CoroNa Green signal compared to control regenerating tail buds (Figure 29).

![Figure 29. TMS Inhibits Sodium Flux in The Bud’s Cells During Tail Regeneration. The first row shows cells positive for CoroNa Green. The bottom row merges the top row image with the normal light illumination image of the worm. A-F) Images show the distribution of intracellular sodium in tail bud region during regeneration.](image)

At 6, 18, and 24 hours following transection, monensin was added to regenerating head and tail body fragments. Although the same concentration of sodium and monensin was added to all different times point, the highest concentration for sodium was seen at 24 hr for both head and tail around the cut site (Figure 30). This result suggested that sodium ions are more required at 24 hr than at earlier time points.
Figure 30. Monensin increased Sodium Flux in The Bud’s Cells During Head and Tail in Early Stages of Regeneration. The left column of each panel shows cells positive for CoroNa Green, which binds to sodium ions. The right column of each panel merges the left column with normal light illumination image of the worm. A-C) Images show the distribution of intracellular sodium in head cut site during regeneration. D-F) Images show the distribution of intracellular sodium in tail cut site during regeneration.

Ultrastructure Level

Worms were examined at the ultrastructure level for both control and TMS-treated worms. When viewed using a transmission electron microscope, treated worms showed the presence of lamellar bodies (Schmitz, et al. 1999) (Figure 32).
Figure 31: Scanning Electronic Micrograph. Micrograph of cross section of freeze dried worm showing a lateral giant fiber (1), a medial giant fiber (2), esophagus and intestinal area (3), circular muscles (4), longitudinal muscles (5), coelomic cavity (6), setae (7), dorsal blood vessel (8), and epidermis (9). Total magnification 140,000X.
Figure 32. Transmission Electronic Micrograph Thin Section of Head Regeneration at Day Five. A) Control worm showing (N) nucleus (S) muscles (G) Golgi. Total magnification 3000X. B) Worm treated with tricaine showing (N) nucleus (L) lamellar...
bodies. Total magnification 7000X. Treated worms showed high number of lamellar bodies.
Discussion

Regenerating Body Fragments

*L. variegatus* have a remarkable ability for regeneration that involves both epimorphic and morphalaxic regeneration. Epimorphic regeneration requires dedifferentiation of cells at the injury site and activation of neoblasts to form a blastema containing a population of undifferentiated cells capable of growth and differentiate into new bud tissues. Morphalaxic regeneration alternatively, is a pattern of regeneration that does not involve any differentiation of stem cells but involves the transformation of existing body tissues into newly organized structures with little new cell growth where adult segments transform the fragment to match their new positional identity (Martinez et al., 2004).

Following transection, there is the formation of a blastema, which is a mass of undifferentiated cells capable of growth and differentiation, and which subsequently, gives rise to a segmental bud (Yoshida-Noro et al., 2010; Zoran, 2010). In *Enchytraeus japonensis*, histological study showed that neoblasts (which are putative mesodermal stem cells) and dedifferentiated epidermal and endodermal cells are involved in the blastema formation (Yoshida-Noro et al., 2010). All of these processes are considered to be the epimorphic pattern of regeneration, which involves blastema formation, adding body segments to the fragment by dedifferentiation of existing cells and activation of stem cell populations to differentiate (Martinez et al., 2004).

An example of morphallaxis occurs in central nervous system during neural morphallaxis when the sensory fields transform as to which giant nerve fiber (Medial
Giant Fiber or Lateral Giant Fiber) is activated by tactile stimulation. (Drewes, 2001).

Following regeneration, the neural organization of the giant fiber sensory input has been transformed to that of a different region of the worm from which it originated.

Regeneration of the head segments following amputation produces 7-8 new segments; while the number of regenerated tail segments is dependent on the time following amputation, with more segments being produced at longer time intervals (Martinez et al. 2004).

**Stem Cells (neoblast) location, division and migration during regeneration**

Previous work on cells dissociated from *L. variegatus* at different time points during regeneration used BrdU to label dividing cells, but those results didn’t show extensive cell proliferation until 120 hr into regeneration (Tweeten et al., 2008). Here, Edu labeling was used in vivo and in intact worms through 10 days of regeneration to clearly indicate the number and position of cells proliferating around the body and in the cut site. In intact worms there were proliferating cells scattered nonspecifically around the body of the worms, which suggests that neoblasts in these worms do not have a specific location in the body. Observation of worms through 10 days of regeneration showed that increased proliferation of cells in the bud is required for both head and tail regeneration. Tweeten et al. (2008) used atrunculin B and locostatin, which inhibited cell migration, to show that tail regeneration was inhibited while only partial anterior regeneration was observed. They suggested that head regeneration in *L. variegatus* does not depend on cell migration as completely as tail regeneration, but could be similar to
vertebrate regeneration where dedifferentiation, proliferation, and re-differentiation of cells at the wound site comprise much of the regenerative process. Results collected in my study provide part of the answer to these questions. These results suggest, migration of the stem cells that are scattered around the body in intact worms to the cut site. This supposition is based upon the observation that the number of cells scattered around the body decreased while the number of cells in the bud region increased (Figure 14-20). In contrast, during head regeneration a higher number of cells at the cut site was observed at the initiation of regeneration (6 hr post amputation), which suggests that cells in the cut site goes under de-differentiation. In the tail on the other hand, the distributions and movements of cells through the 7 days suggest cell migration (Figure 14, 17).

Head regeneration from both posterior end fragments and middle section fragments resulted in the formation of 8 body segments containing the mouth by day 6 post transection (Figure 5, 7). On the other hand, the ability of tails to produce new segments differed with more segments being produced from anterior fragments that from middle fragments (Figure 5, 8). Previous study suggested that tail regeneration is dependent on migration of cells to the cut site (Tweeten, et al., 2008). One explanation for the difference of growth in tail buds between anterior fragments and middle fragments is since anterior body fragments regenerate only a new tail, and there is a head already present, and that all neoblasts cells available will migrate to the cut site but fragments from the middle section are regenerating both a new head and a new tail at the same time (Figure 4) so fewer cells are available to migrate to the two regenerating sites resulting in slower segmental regeneration in the middle fragments. The results from EdU staining
for middle fragments regenerating both a head and a tail at the same time are compatible with this explanation (Figure 20) by showing that proliferating cells migrating to both the head and tail cut sites to form blastemas. Although, the head blastema was completely formed by 48 hr similar to head blastema from posterior fragments (Figure 20B, 21B), tail blastemas for middle fragments didn’t form completely until 72 hr into regeneration (Figure 20 C), unlike tail blastemas from posterior fragments which completely form by 48 hr (Figure 18E).

In the Tweeten et al. (2008) study, they suggested that head regeneration depends on dedifferentiation, proliferation, and re-differentiation of cells at the wound site. Since the results of my study showed no difference in the head regeneration from posterior fragments and middle section fragments which supports the idea that head regeneration is dependent on dedifferentiation, proliferation, and dedifferentiation of cells at the wound site without depending on cell migration.

**Sodium Distribution During Head and Tail Regeneration**

The results from EdU labeling and CoroNa Green for cells in the bud region during regeneration for head and tail regeneration showed a relation between the number of dividing cells and sodium-positive cells. For example proliferating cells in head regeneration from both posterior and middle fragments were more prominent from day 3 to day 5 (Figure 21), which is also the time showing the most cells positive for sodium indicator in the same body parts (Figure 26), suggesting that sodium is required for cell division during this state of regeneration. Sodium in cells in the bud region of head
middle fragments was higher than the concentration of sodium in cells in the bud region of head posterior fragments. As mentioned earlier, head and tail regeneration from middle fragments shared cell migration, which means cells in the head bud region of middle segments go through cell dedifferentiation to compensate for the shortfall of stem cells. On the other hand, cells in the bud region for both body fragments didn’t show differences in sodium concentration (Figure 27).

**Effects of Tricaine Methanesulfonate on Regeneration**

Tricaine is a local anesthetic of the amino amide class, which is known to inhibit certain mammalian Na\(_V\) channels (Seetharamaiah, et al. 2014). The *Lumbriculus variegatus* Na\(_V\) channels targeted by TMS have not been specifically studied; however, the common mode of action across diverse phyla supports the supposition that TMS acts through Na\(_V\) channels in *Lumbriculus*. Recent work has shown that sodium transport via Na\(_V\) is required during the initial stage of tail regeneration in *Xenopus* embryos (Tseng et al., 2010). The effects of TMS on head and tail regeneration in *Lumbriculus variegatus* showed different effects on head regeneration and tail regeneration, which means that the requirements for sodium transport during regeneration is different between head and tail regeneration.

Head regeneration for worms treated with TMS showed defective morphology compared to control head regeneration (Figure 7 A-D). Regenerating heads in TMS-treated worms were able to grow in length but showed axial asymmetry indicating that dividing cells are more prominent on one side of the body. The differential distribution of
sodium during head regeneration in TMS-treated worms suggests a mechanism for the morphological abnormalities (Figure 21, 28).

TMS- treated worms showed more prominent reductions in tail regeneration than in head regeneration, with little growth occurring and no well-defined formation of segments. Brackenbury et al. (2008) suggested that Nav α and β subunits play an important role in regulating migration during normal postnatal development of the CNS as well as during cancer metastasis. As I suggested earlier that tail regeneration is more dependent on cell migration, therefore inhibition of Na, via TMS results in a major reduction of regeneration in the tail more than the head.

To define the temporal effects of TMS and the requirement for NaV-mediated sodium transport during regeneration, worms were treated with TMS at different times post transection. The results showed that worms treated with TMS at 48 hr after transection showed less significant reduction in regeneration for both heads and tails than worms that treated from 0 hr to 24 hr following transection. These results suggest that sodium transport is principally required during the initial stages of the bud formation, which occurs prior to 48 hr of regeneration. This was especially true for head regeneration. In contrast, tail regeneration required cell migration beyond 48 hr, which might be one reason why regenerating tails from anterior fragments produce a higher number of segments than tail regenerating from middle fragments as mention earlier.

The effects of on cell division during regeneration was examined via EdU staining. Comparison of cell proliferation between control worms and worms treated with TMS for both heads and tails bud regions during 7 days of regeneration showed
significant reductions in cell proliferation. Notably, no significant change in proliferation was seen in other regions of the body between untreated and treated worms. These results suggest that Na\textsubscript{v}-mediated sodium flux is required for specific up-regulation of proliferation in the bud region but not for general cell division.

**Monensin treatments**

Monensin is an ionophore antibiotic that is capable of collapsing Na\textsuperscript{+} and H\textsuperscript{+} gradients. Monensin binds a sodium cation as a salt by losing a proton from its carboxyl group and then moving the cation across the cell membrane. Upon releasing the sodium cation, the carboxylate anion is protonated. Differences in the cation concentration outside and inside the cell power this process. This mechanism of transport is electroneutral (Łowicki et al. 2013; Huczyński et al. 2012). The net effect for monensin is a trans-membrane exchange of sodium ions for protons. However, the interaction of an ionophore with biological membranes, and its ionophoric expression, is highly dependent on the biochemical configuration of the membrane itself (Mollenhauer, 1990).

Results in this study showed that the addition of monensin with or without the addition of sodium gluconate, immediately upon transections showed a decrease in regeneration (Figure 10, 11). Adams, et al. (2007), they showed that H\textsuperscript{+} flowing into the *Xenopus* tail cells during regeneration is crucial for regeneration especially at 6 hr post transection and they were able to rescue regeneration of refractory tails by induction of H\textsuperscript{+} pumping. Since monensin mechanism of transporting the Na\textsuperscript{+} ions through the cell membrane affects also the H\textsuperscript{+} gradients, this could be the reason of the reduction in
regeneration that was observed in worms treated immediately upon transection with monensin. When monensin was added to both control worms and TMS-treated worms for only 45 minutes and post 18 hr of transection, although the results was not significant they showed clearly some type of regenerative outgrowth especially in the tail bud when compared to control (pond water) and TMS-treated worms, respectively (Figure 12, 13).

The CoroNa Green results, showed that monensin-treated worms showed higher concentration of Na\(^+\) in cells in the bud and around the cut site (Figure 30) compared to control worms and TMS-treated worms.
References


