Exploring the neural control of axolotl limb regeneration

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A dissertation submitted to

The Faculty of
the College of Science of
Northeastern University
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

April 6th, 2017

Dissertation directed by
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Acknowledgements

As I look back on my past five years at Northeastern University, I recognize that I would not even be in the position to write this dissertation without the help of a wide array of remarkable people. In truth, so many people have helped and supported me over the years that it would be difficult to list them all, and I am truly grateful to every person who offered their advice, support, or mentorship throughout my career.

I would like to start by thanking my advisor, Dr. James Monaghan. I simply could not ask for a better mentor, his choice of football teams notwithstanding, and I fully recognize the extent of my fortune in joining his lab right at its inception. I have learned so much during my dissertation that feel I have emerged from the experience not just as a far more educated student, but as a more confident and responsible person. I owe the majority of this growth to Dr. Monaghan’s remarkable patience, intelligence, and mentorship. Witnessing the evolution of the lab from a few original members (and four axolotls) has been an incomparable experience, and I am forever grateful that I was able to take part in it. I thank Dr. Monaghan for providing us all with an outstanding environment in which to work and learn, and I will always treasure the many adventures I had in this lab- except for all those hours of bead implantation surgeries, which I would rather forget entirely. I would also like to thank Dr. Dori Woods, Dr. Gunther Zupanc, and Dr. Fred Davis, all faculty members of the Northeastern University Department of Biology on my committee. I truly appreciate their advice and suggestions for the direction of my research, all of which were vital for helping me stay on course. I would also like to thank the fifth member of my committee, Dr. Jessica Whited of the Brigham Regenerative Medicine Center, both for her mentorship and her collaboration on many of these research efforts.
I have formed many lasting friendships and connection during my time in the Monaghan lab. Polina Freitas deserves more thanks than I can possibly espouse here, not just because of her friendship and contributions to the research but also because of her immaculate baking skills and incredible cleaning abilities (particularly with regard to my perpetually disastrous desk), which have kept me far more sane and organized than I would have been otherwise. I’d like to thank Piril Erler as well, without whom I definitely would have ended up lost and alone in the streets of Napoli, and Alex Mulcahy for her contributions to the following work. While there is no space for me to acknowledge every one of the undergraduates and temporary members of the Monaghan lab who helped me throughout the years, I would like to thank Matthew Nguyen and Pankhuri Singhal in particular for their enduring support and friendship.

Despite all of this encouragement from the world of research, I would not have made it this far without significant support from those outside of the lab as well. To this end, I would like to thank my amazing mother, Martha Hagopian, who has been an unwavering role model throughout my life and who worked unbelievably hard to make sure I was able to attend the undergraduate institution of my dreams. I would also like to wholeheartedly thank Sam Huntress, my fiancé, who has supported me unwaveringly for many years now even despite all my late nights and occasional tirades. I would also like to thank the many good friends who issued no complaint when I had to cancel plans because of late-night surgeries, my undergraduate mentors Dr. David Gapp and Pearl Gapp, and Mr. Cormier, who knew I’d be writing this dissertation more than a decade before I did. Like all scientific endeavors, this was a collaborative effort and bears the signature of every person who had aided me along the way. Irrespective of their inclusion above, I would like to express my heartfelt gratitude towards each and every one of them.
Abstract of Dissertation

Salamanders are capable of feats of tissue regeneration which are unmatched by other tetrapods. In contrast with mammals and virtually all other vertebrates, salamanders, among which the Mexican axolotl (Ambystoma mexicanum) is the most well-studied, can perfectly regenerate damaged organs and amputated structures. Chief among these astonishing capabilities is the phenomenon of axolotl limb regeneration. Axolotls are capable of fully regenerating amputated limbs throughout the entire course of their lives. This regeneration is perfect and demonstrates the complete return of both the original structure and function of the limb. However, although salamander limb regeneration has been studied in a scientific context for centuries, little is understood about the molecular basis of the process. What has been known for nearly two hundred years is that peripheral nerves are essential for axolotl limb regeneration. Denervation of the axolotl limb prevents the formation of the post-amputation proliferative mass called the blastema and thus completely inhibits regeneration, and the molecular biology of this nerve dependence serve as the foundation for the work described here. We have discovered that the nerve dependence of axolotl limb regeneration, a longstanding puzzle for researchers in the field, may in fact be the result of a combination of factors. Our published work demonstrates the importance of Neuregulin-1 (NRG1), a nerve-derived mitogen, for blastema formation and growth. We have found that NRG1 is localized to the regenerating blastema, capable of rescuing regeneration to digits in denervated limbs, and inhibition of this signaling pathway inhibits regeneration. While this work validates the longstanding neurotrophic hypothesis of axolotl limb regeneration while also describing the first protein known to demonstrate these characteristics in the regenerating axolotl limb, we have additionally found that there is a second component to nerve dependence. Our unpublished work, currently in preparation for submission, has shown
that nerves are damaged during denervation and prevent the formation of a regeneration-permissive cellular environment by means of the secretion of inhibitory factors. Specifically, we have found that implanting axotomized nerve bundles into the wound site of amputated limbs slows down or blocks blastema formation and may lead to highly aberrant limb patterning. These effects were mitigated via overexpression of NRG1, suggesting a link between the two hypotheses by indicating that NRG1 is capable of reversing the inhibitory effects of damaged nerves. These novel findings grant us deeper insight into the molecular mechanisms utilized by a highly regenerative animal, one which may hold the key to unlocking similar abilities in less-capable organisms. Therefore, our work, both as described in this dissertation and continuing into the future, may ultimately inform future studies of regenerative medicine in humans.
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<td>AB</td>
<td>accessory blastema</td>
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<td>ABM</td>
<td>accessory blastema model</td>
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<td>AL</td>
<td>accessory limb</td>
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<td>ALM</td>
<td>accessory limb model</td>
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<tr>
<td>APBS</td>
<td>amphibian phosphate buffered saline</td>
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<td>areg</td>
<td>amphiregulin</td>
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<td>BP</td>
<td>brachial plexus</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<td>DPA</td>
<td>days post-amputation</td>
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<td>DPI</td>
<td>days post-injury</td>
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<td>DRG</td>
<td>dorsal root ganglia</td>
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<td>EGFR</td>
<td>epidermal growth factor receptor</td>
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<td>FGF</td>
<td>fibroblast growth factor</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<td>IHC</td>
<td>immunohistochemistry</td>
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<td>NRG1</td>
<td>Neuregulin-1</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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Chapter 1: Introduction to the axolotl

The Mexican axolotl (*Ambystoma mexicanum*), a large aquatic salamander capable of regenerating tissues and structures throughout adulthood, is an invaluable model for the study of complex tissue regeneration. Capable of perfectly regenerating amputated limbs, salamanders are the tetrapods most closely related to mammals which demonstrate this remarkable regenerative ability, and thus the study of these animals may one day inform human regenerative medicine. The axolotl has become the favored salamander for regenerative research because it is comparatively easy to breed and maintain in a laboratory setting. However, while the axolotl’s capabilities have been a source of scientific study and fascination for centuries, the molecular basis of complex salamander tissue regeneration remains largely unexplored. The purpose of my work, described in the following chapters, is to elucidate the molecular mechanisms of axolotl limb regeneration. More specifically, I will examine the role of peripheral nerves during this process, in an effort to determine why the presence of intact peripheral nerves is necessary for axolotl limb regeneration. This work will be presented as both published and drafted manuscripts. The first three chapters are published first-author reviews which cover topics ranging from axolotl anatomy to the molecular mechanisms of nerve dependence, while the next two chapters consist of original research, including one published paper and one paper that is currently in preparation. Finally, all conducted research will be summarized and integrated in the concluding chapter, which will address future avenues of study.

The following publication was written in 2015 and serves as an introduction to the axolotl as well as a guide towards its laboratory maintenance and care. Published as a chapter in the book *Salamanders in Regeneration Research*, presented by Springer Protocols in the Methods in Molecular Biology series (Farkas & Monaghan, 2015), the aim of this chapter was to describe
the history of the axolotl in addition to its physiology and the basics of its husbandry. This chapter thus serves as an introduction to my further work and is reproduced in its entirety below.

**Housing and Maintenance of *Ambystoma mexicanum*, the Mexican axolotl**

Johanna Farkas and James R. Monaghan

**Summary**

The aim of this paper is to assemble a significant amount of information on *Ambystoma mexicanum*, the axolotl salamander, to assist in the basic knowledge needed to raise, breed, and study most aspects of axolotl biology. It is important to understand the basic biology of the axolotl in order to make informed decisions on their proper care and use in experiments. Therefore, we will provide the necessary information to the non-herpetologist that will assist in their study of this unique and fascinating animal. We also aim to provide a resource on the general anatomy, behavior, and experimental tips specific to the Mexican axolotl that will be of use to most axolotl laboratories. Axolotls have been actively researched since the 1860’s, giving testament to their relatively straightforward maintenance and their versatility as an animal model for development and regeneration. Interest in using the axolotl in laboratory research has grown tremendously over the past decade, so dedicated resources to support the study of this species are needed and encouraged.

1. **Introduction**

1.1 **Taxonomy, habitat, and the lab strain**

*Ambystoma mexicanum*, commonly named the axolotl (Fig.1.1), are members of the order Urodela (*oura* tail + *delos* evident, also called Caudata) or tailed amphibians, which constitute ten extant families of salamanders found mainly across the temperate regions of the northern hemisphere. Axolotls belong to the family Ambystomatidae, genus *Ambystoma*, which are
commonly called mole salamanders and are comprised of approximately 30 species found across North America from southern Mexico to southern Alaska. There are seventeen Mexican ambystomatid salamander species that inhabit the mountains of Central Mexico. Five of these species are primarily or obligatorily neotenic, meaning that they do not undergo metamorphosis and can breed in the adult larval form (Shaffer, 1989). Axolotls are endemic to the Lake Xochimilco area in the Valley of Mexico, which has been reduced over the centuries to a ~40 km² area of artificial canals just outside the city limits of Mexico City (Globus et al., 1991b). This high altitude lake system (~2,200 meters above sea level) has been inhabited for centuries, most notably by the Aztecs. This is where the name axolotl originates, as the animal was named after the Aztec god Xolotl (Thorsen & Hale, 2007). The water conditions of Lake Xochimilco between 1978 and 1988 were estimated to be between 16-20°C, pH 7.4-8.0, with a conductivity between 975-1650 microSiemans (μS)/cm (Shaffer, 1989). Today, axolotl are critically endangered and are on the brink of extinction due to habitat loss, the introduction of invasive species, and shifts in water quality (Onda et al., 1990). It has been estimated that densities were at 6000 ind./km² in 1998, 1000 ind./km² in 2000, 100 ind./km² in 2008 (Globus et al., 1991b), and only a few axolotls were cited in the lake system after months of surveying in 2013 (Kumar et al., 2007a). Unfortunately, the majority of axolotls today are found in aquaria and laboratories around the world.

The modern axolotl strain used in most laboratories is a highly inbred population that most likely arose from a donation of seven wild axolotls (six wild-type and one white mutant) between 1863-1866 to the Paris Natural History Museum (Ferretti & Brockes, 1991). In fact, most modern day laboratory axolotls likely have a direct lineage to these founders, and all white mutants are descendants from this single white animal (Stocum, 2011b). A few wild-caught
axolotls were introduced into the colony strain in the 1960’s including an albino tiger salamander
(*Ambystoma t. tigrinum*) (Poss et al., 2002a), but overall the present day laboratory strain is
likely one of the most long-running inbred strains of any laboratory species. The 150-year
history of laboratory breeding seems to have selected against spontaneous metamorphosis
(currently <1% frequency), as it is more prevalent in wild strains than the lab strain (Stocum,
2011b; Van Arsdall & Lentz, 1968). The most extensive colony of laboratory axolotls is
maintained at the Ambystoma Genetic Stock Center (AGSC) at the University of Kentucky
(www.ambystoma.org), which is a continuation of the Indiana University Axolotl Colony
initiated by Humphrey R.R. in 1957 (Jaźwińska et al., 2007). Over the past 50 years, the Axolotl
Colony has provided the majority of axolotls and housing information to labs worldwide.
Valuable information on housing, breeding, and diseases of axolotls can be found at
www.ambystoma.org as well as in the archives of the Axolotl Newsletter.

1.2 Laboratory research using the axolotl

Axolotls have classically been used in developmental biological research for practically every
organ system due to their large egg size, external development, reliable breeding, acceptance of
embryonic and adult tissue grafts, and large clutch sizes (Voss et al., 2009). Historically, both
newts and axolotls have been the primary types of salamanders used in lab research, but the
unique advantage of axolotls is that they can be easily bred in a lab environment in the long term.
What truly sets both newts and axolotls apart from other animal models is that they are our
closest relatives that display a wide range of striking regenerative capabilities. In fact, axolotls
can regenerate and recover from virtually any injury that does not kill them. Regeneration has
been observed in parts of the heart, the tail, the jaw, the spinal column, the gills, the brain, and
entire limbs (Fig 2). This regrowth occurs without scarring and with full restoration of function.
It can also presumably reoccur an indefinite number of times without any loss of fidelity, although a careful analysis of this point is needed in axolotls. Regeneration can be induced throughout all stages of life, although the process is faster in larvae and less reliable in animals that have been forced to undergo metamorphosis (Monaghan, 2014; "R: A language and environment for statistical computing.").

Other amphibians besides axolotls are studied for their regenerative abilities. Tadpoles of the African clawed frog *Xenopus laevis* can regenerate their tails and spinal columns, while certain species of newts (*Pleurodeles waltl, Notophthalmus viridescences*, and *Cynops pyrrhogaster* are the most commonly studied species) can regenerate appendages and organs. Despite these similarities, the axolotl is not closely related to these species. As a member of the order Anura, *X. laevis* is only distantly related to urodele salamanders with a common ancestor approximately 260 million years ago (Zhang & Wake, 2009), a divergence emphasized by the fact that *X. laevis* loses its regenerative abilities in late larval stages. Furthermore, although newts and axolotls are both urodeles with superficially similar anatomy, they diverged from a common ancestor at least 145 million years ago (Zhang & Wake, 2009), and recent studies have shown that newt regeneration and axolotl regeneration differ in both mechanism (Blassberg et al., 2011; Kumar et al., 2010; Sandoval-Guzman et al., 2014) and recovery after denervation (Liversage & McLaughlin, 1983). Consequently, caution is advised when attempting to compare axolotl protocols and findings with those of other amphibian models.

### 1.3 Gross anatomy

Unlike the majority of urodeles, axolotls are obligate paedomorphs which do not undergo metamorphosis unless it is artificially induced via the addition of thyroid hormone to their environment (Page & Voss, 2009). Consequently, they grow to be large fully aquatic adults and
maintain the feathery external gills characteristic of ambystomatid larvae throughout their lives (Fig 1; Fig 2). Males reach sexual maturity, indicated by a blackening of the nails, at around ten months of age while females tend to mature slightly later from 12-18 months. Sexual dimorphism is moderate but visible, as males are slimmer and longer than females and exhibit a cloacal bulge (Fig 1.2A). Sexually mature females are also more rotund due to their egg supply. While the lifespan of wild axolotls remains unknown, they generally live between 10 and 15 years. Animals can be induced to undergo metamorphosis, which become terrestrial and strongly resemble adult tiger salamanders (*Ambystoma tigrinum*). Axolotls are in fact closely related to tiger salamanders and are capable of crossbreeding with this species in additional to various other ambystomatid salamander species (Voss & Shaffer, 1996). Studies of *A. tigrinum* have thus provided extensive insight into the anatomy and development of *A. mexicanum* (Francis, 1934).

Although the overall body plan of the axolotl is considered to be among the most primitive of the tetrapods, these salamanders nevertheless share a number of basal characteristics with other vertebrates and have proved useful for the study of many different systems. Aquatic and lacking a middle ear structure, axolotls rely on olfaction far more than audition when they are searching for food. The urodele olfactory system is quite complex and allows the animal to discriminate between very similar odorants and chemicals. Because their olfactory epithelium and olfactory bulb are large and easily-accessible, axolotls were a favored model for the study of olfaction and neuronal signaling during the 1970’s and 1980’s (Getchell, 1977; Mackay-Sim & Shaman, 1984; Mackay-Sim et al., 1982). Axolotls possess true teeth and a calcified skeleton with cartilaginous joints that are anatomically similar to mammalian joints (Cosden et al., 2011; Lee & Gardiner, 2012b)
Although their eyesight is poor and their vision is largely limited to the detection of movement, the axolotl retina displays the layered structure typically seen in vertebrates. In fact, many early studies of retinal intracellular signaling were conducted in salamanders because of their large and easy-to-access retinal cells (Dvorak, 1984; Grabowski & Pak, 1975; Waloga & Pak, 1978). Axolotls can detect ultraviolet light, and their UV-sensitive photoreceptors express three different opsins (Isayama et al., 2014; Makino & Dodd, 1996). Furthermore, like fish and other aquatic amphibians, axolotls possess a lateral line system that is used to detect both electrical currents (via ampullary organs located on the head) and water movement (via mechanoreceptive neuromasts that run along the side of the animal). The lateral line develops from neurogenic placodes, and this developmental process may provide insights into the evolution of vertebrate sensory systems (Smith, 1996). However, the study of the axolotl’s development and evolutionary history has been complicated by its massive genome. Consisting of around $32 \times 10^9$ basepairs located across 14 haploid chromosomes (Straus, 1971), the axolotl diploid genome is among the largest of all tetrapods and has yet to be fully sequenced. Our understanding of this animal is sure to increase rapidly as genetic technology advances and more techniques are adapted for use with salamanders.

1.4 Structure of the circulatory and respiratory system

Axolotls possess a three-chambered heart consisting of two atria and one ventricle. As blood leaves the ventricle it can pass to either the pulmonary arteries or through an aorta that leads to the rest of the body. Oxygenated blood leaves the lungs and is pumped back into the lone ventricle of the heart, where it mixes with deoxygenated blood that has already circulated through the body. This three-chambered system is consequently less efficient than the four-chambered system seen in mammals and birds, as tissues are nourished by blood that is not
saturated in oxygen. The number of aortic arches can vary greatly between and within different urodele species, but axolotls always have four aortic arches (Putnam & Parkerson, 1985). Like all amphibians, axolotls are poikilothermic and their heart rate is strongly influenced by the temperature of their environment. Hematopoeisis arises from the adult liver and spleen (Smith et al., 2006) and their erythrocytes are very large, nucleated, and strongly autofluorescent. Injury induces rapid vasoconstriction to prevent excessive blood loss, and clotting occurs very quickly even after injury to major arteries. As a result, axolotls are at only minimal risk of bleeding to death during and after surgery. They are a fairly popular model for the study of cardiac development because they can exhibit a recessive mutation, dubbed \( c \), that is cardiac lethal within two weeks after hatching. The hearts of \( c/c \) mutants do not beat, and studies have found that these mutant hearts are largely differentiated but lack tropomyosin (Erginel-Unaltuna et al., 1995) and organized sarcomeres (Lemanski et al., 1997).

Aquatic throughout their lives, axolotls utilize multiple strategies for obtaining oxygen from their environment. They exchange oxygen with their three paired external gills and can respire through their skin via cutaneous gas exchange. However, axolotls also possess rudimentary lungs and may obtain at least 40-60% of their oxygen through surface breathing (Whitford & Sherman, 1968). These lungs are elongate and transluscent in appearance, and run parallel to the spinal column for virtually the entire length of the body cavity (Fig. 1.2B). Very little research on axolotl respiration has been conducted, but previous studies of tiger salamander larvae have provided likely insights into the mechanism of this process. These larvae inhale using a two-stroke buccal pump system that mixes expired and fresh air in the buccal cavity before pumping it into the lungs. Exhalation is active and very rapid, minimizing the amount of fresh air that is expelled through the mouth and gills. This system results in ventilation
efficiency that is comparable to that of mammalian ventilation (Brainerd, 1998). Axolotls housed in hypoxic tanks make frequent trips to the surface to breathe (McKenzie & Taylor, 1996), which can increase the probability that they will swallow air and cause the formation of an air bubble within the body that can disrupt the animal’s locomotion and feeding. Tip: Because of their numerous options for respiration, axolotls can survive for hours outside of their tanks so long as they are not allowed to desiccate. This ability proves useful for the purpose of surgical procedures, as axolotls will heal very quickly if kept moist and left sedated after surgery.

1.5 The immune system

Axolotls have a very primitive acquired immune system and are generally described as immunodeficient. They do not induce a humoral response to soluble antigens (Ching & Wedgwood, 1967), they produce just two classes of immunoglobulin, and they generate antibodies to antigens extremely slowly if at all (Charlemagne, 1979). This immunodeficiency is common in urodeles and has worked to the advantage of researchers, as axolotls do not reject tissue from other salamanders and will even readily accept tissues (Sessions et al., 1989) and tissue primordia (Harris & Cole, 1984) from other amphibian species such as *X. laevis*. Creative grafting experiments using embryos have done much to elucidate the development of axolotls, while grafting of larval or adult tissue has uncovered some of the mechanisms of regeneration. The recent production of GFP-expressing axolotls has opened up a new avenue of grafting possibilities, and researchers have already begun grafting GFP tissues to white animals in order to reveal the basis of cellular plasticity and positional memory during regeneration (Sobkow et al., 2006b).

Despite these deficiencies in the acquired immune system, the axolotl is still resistant to
bacterial infections. This is likely due to their mucus coat and fairly robust innate immune system, which represent the main line of immune defense for urodeles. Abundant neutrophils and macrophages rapidly engulf and destroy foreign invaders, while antimicrobial peptides provide an additional layer of defense (Froese et al., 2005). Past the age of two weeks, axolotls are at moderate to minimal risk of bacterial infection even after multiple surgeries. However, they are still susceptible to fungal and viral infections and can acquire bacterial infections after chronic stress. Exposure to Ambystoma tigrinum virus (ATV) can devastate both wild and laboratory populations, with mortality rates potentially exceeding 90% (Chinchar et al., 2009). This extreme susceptibility is likely due to a lack of lymphocyte proliferation upon exposure to the virus (Cotter et al., 2008). Another curiosity of the axolotl immune system is the fact that macrophages seem to be essential for regeneration, as early ablation of macrophages completely inhibits blastema formation and results in excessive collagen deposition after limb amputation (Godwin et al., 2013).

Intriguingly, although they may undergo high levels of cellular proliferation in multiple tissues throughout their lives, axolotls are remarkably resistant to cancer. Repeated studies of various urodeles have failed to induce cancerous growth in regenerating tissues even upon administration of carcinogens (Tsonis & Eguchi, 1981; Zilakos et al., 1996), although spontaneously-occurring tumors have been described in veterinary literature (Harshbarger et al., 1999; Shioda et al., 2011). This curious resistance to malignant growth even during extreme cellular proliferation remains largely unexplored.

1.6 Pigmentation and structure of the epidermis and nervous system

Wild-type axolotls are a mottled gray/brown/olive-green color, and this coloration arises from a combination of three different neural crest-derived pigment cells. Black melanophores
are the predominant chromatophores in adult wild type animals, while yellow xanthophores are more abundant early in development (Fig.1.1)(Frost et al., 1984). Tip: Shiny iridophores reflect light and are strongly reflective under fluorescent imaging. White leucistic (white mutant; d/d) axolotls are the result of a recessive mutation in an unknown gene. They are often times preferred over wild type animals for research purposes, as pigment can negatively affect histological staining or fluorescent imaging. Leucistic animals are descendants of a single founder white axolotl that was donated to Auguste Duménil in 1866, hence the genetic designation d (Sugiyama et al., 2009). Leucistic larvae are lightly pigmented dorsally, but lose almost all pigment shortly after hatching due to a lack of pigment cell migration to the flank of the animals (Nechiporuk & Keating, 2002). In contrast, albino animals completely lack melanin and have a yellow appearance due to an overabundance on xanthophores (Fig.1.1). Tip: although albino eggs are difficult to inject due to their lack of pigment, they are very useful for whole-mount embryonic staining methods. Adult leucistic salamanders can be easily distinguished from albinos by their black eyes.

The adult axolotl epidermis is similar to other larval amphibians and does not contain the distinct cornified layer (stratum corneum) that mammals or metamorphosed amphibians possess (Seifert et al., 2012c). It is highly vascularized in order to facilitate efficient cutaneous gas exchange and is coated with mucus secreted by epidermal Leydig cells and dermal mucous glands that help retain moisture and withstand microbial threats. Leydig cells are large, club-shaped, and packed with secretory granules (Fig.1.3A, B). As they fill with these granules, they rupture and release mucus into the intracellular space where it can then escape via pores onto the epidermal surface (Jarial, 1989). Tip: this mucus coat is very sticky and makes for an effective natural adhesive during surgery. Epidermal healing and regeneration is completely scar-free
and occurs without extensive long-term fibrosis (Seifert et al., 2012c).

1.7. Regeneration and the nervous system

Limb regeneration is the classic paradigm of complex tissue regeneration and is currently the primary focus of laboratory research on the axolotl (Fig 2B). Axolotls regenerate limbs by generating a mass of highly proliferative cells called a blastema at the distal tip of a limb stump (Fig 3C). After amputation, locally-derived lineage-restricted progenitor cells or dedifferentiated cells proliferate and recapitulate developmental processes in order to regrow the missing portion of the limb (Mescher & Munaim, 1984). As long as animals are well cared for, limb regeneration is a robust, dependable assay. A juvenile animal (8.5-10cm SVL) will reach the differentiation stage of regeneration around approximately 32 days post amputation, but will not replace 100% of the missing limb for at least 100 days post amputation. Three-month old axolotls (~5cm SVL) will reach the differentiation stage of regeneration at approximately 22 days post amputation and will replace 100% of their limbs by approximately 66 days post amputation (Monaghan, 2014).

Perhaps because axolotls are a neotenic member of an evolutionarily primitive order, their brains are very simple and share several similarities with the mammalian embryonic brain (Fig.1.3D). The axolotl brain is relatively flat and elongated with clear delineations between the telencephalon, mesencephalon, and rhombencephalon (Fig.1.2B). The olfactory bulbs are large but the optic lobes are small and poorly-separated, and the cerebellum is relatively undersized and weakly-developed as well (Francis, 1934). The cerebellum contains the only neurons in the axolotl central nervous system that are not paraventricular- all other neurons remain stationary and do not migrate from the germinal site during development (Harris, 1989) (Fig.1.3D).

Though less is documented about the terrestrial axolotl brain, it is known that the brain
undergoes structural changes during the metamorphic process, particularly in the optic lobes as they grow to accommodate the animal’s new mode of binocular vision (Stirling & Brandle, 1982). This suggests that the axolotl brain remains plastic and capable of drastic change throughout the animal’s lifespan, possibly indicating that axolotls would make a favorable model for the study of neural plasticity. Although seemingly possessed of limited intelligence, laboratory axolotls will learn to associate humans with food and will move to the front of their tank in anticipation of feeding. Few studies on axolotl learning have been conducted, but classical conditioning studies have been performed on tiger salamanders and seem to be most effective when the conditioned stimulus is olfactory in nature (Dorries et al., 1997). Tiger salamanders have also been trained to complete a T-maze test, a feat which occurs more rapidly and reliably in adult animals than in larvae (Schwartz & Cogan, 1977).

Unlike mammals, which possess only limited regenerative potential in the central nervous system, the axolotl CNS can recover from extensive injury. Large portions of the axolotl brain can regenerate fully after injury, and studies have found that they can even recover from complete lobectomy (Kirsch & Kirsch, 1964; Richter, 1968). Neuronal proliferation has been observed all along the ventricular zone of adult brains (Maden et al., 2013). Axolotls are also capable of fully recovering from spinal crush and regenerating their tail, spinal column included, after amputation. Both of these regenerative processes occur without the formation of a glial scar in salamanders (Okamoto et al., 2007; Parish et al., 2007), which in mammals is thought to inhibit axonal growth after CNS injury. Precisely why the CNS of these animals can heal without glial scarring remains under investigation.

The peripheral nervous system of the axolotl is simple but organized in a manner similar to other tetrapods. The axolotl PNS is of particular interest to researchers because of the
phenomenon of nerve-dependent regeneration. If a limb is amputated and then denervated within approximately seven days—easily accomplished by severing two of the three major nerves at the brachial plexus—the blastema does not form and regeneration does not occur. The cause of this nerve dependency is still under investigation, although it may be due to a loss of nerve-secreted mitogenic factors that are essential for kickstarting the regenerative process. Axolotl peripheral nerves are myelinated and generally easy to find and sever, particularly in developing animals. However, they begin to regrow within ten days and must be periodically re-severed in studies that last longer than this span. *Tip: denervation becomes successively more challenging over time, and consequently it is difficult to maintain a fully denervated state for longer than approximately twenty days.*

1.8 Reproductive system structure

Axolotls reproduce sexually and fertilize internally. Males lay spermatophores which are then taken up and dissolved by the female in the spermatheca, allowing spermatozoa to be stored in the cloaca until it is time for spawning to occur. As eggs leave the oviducts and enter the cloacal chamber, spermatozoa come into contact with the egg and more than one sperm enter the egg cytoplasm (Whitehead et al., 2005). Eggs are usually laid in strings and are protected by a thick coating of sticky jelly, which must be removed prior to embryonic injections or grafting experiments. Though spawnings vary considerably in size, most females will lay between 200 and 1000 eggs per spawn. Breeding ease and efficiency in the laboratory peaks in the spring and decreases throughout the summer, suggesting that axolotl breeding is seasonal even in animals which have been removed from their natural environment. This seasonality is probably linked to spermatogenesis, which typically begins in early summer and results in the deposition of sperm into the vas deferens during the winter (Armstrong, 1989). Some labs have attempted to
overcome this seasonal impediment by hormonally inducing ovulation with injections of human chorionic gonadotropin (hCG) (Armstrong & Duhan, 1989). However, this technique does not seem to affect the rate of spermatophore deposition, which remains the limiting step in this process.

Despite the fact that axolotls are commonly and reliably bred in the laboratory setting, much remains unknown about the structure and development of their reproductive system. It is known that axolotls share a conserved inductive mechanism of germ cell development with mammals, rather than the oocyte derived germ plasm observed in *Xenopus laevis* and zebrafish (Chin & Yeong, 2010). Structural anatomy of the axolotl ovary has yet to be fully described or characterized, though it has been found that adult females constantly undergo oogenesis and thus their ovaries contain oocytes at all stages of maturation and development (Beetschen, 1989). The axolotl ovary can take up a considerable portion of the peritoneum and resides next to the fat bodies (Fig.1.2B). Although breeding fidelity tends to decrease over time, oogenesis never halts completely. Therefore, although few studies of axolotl ovarian development and anatomy have been conducted, the animal remains an intriguing model of ovarian regeneration and long-term adult oogenesis.

Slightly more is known of the male reproductive system. The axolotl testis is comprised of a variable number of lobes, and older animals tend to have increased numbers of these lobes. Lobes are made of lobules, which resemble sacs and are themselves composed of small spermatogonial cysts, each of which arose from a single stem cell (Armstrong, 1989). As noted previously, spermatogenesis begins in the early summer after old cysts from the previous reproductive cycle have been broken down and replaced. By August the testis is swollen and heavy, and sperm are released into the vas deferens in the following months as cysts rupture and
then degenerate. Not all individuals adhere strictly to this seasonal pattern, and there is even some variation within a single testis as cysts may mature at different rates. Consequently, an axolotl testis is likely to exhibit cysts at many different stages of development.

2. Materials

2.1 Housing

1. A windowless (Note 1) or blacked-out room held at a constant temperature of 16-20°C (Note 2).
2. An automatic light timer which controls lighting conditions for a 12L:12D light cycle (Note 3).
3. Deionized water or dechlorinated tap water supplemented with a balanced salt solution at a pH between 7.0 and 7.5. For example: 40-50% Holtfreter’s solution, which has a conductivity of approximately 2300 μS/cm (NaCl = 30mM, MgSO₄·7H₂O = 0.4mM, CaCl₂ = 0.45mM, KCl = 0.33mM) (Note 4).
4. Water can be stored in a recirculating 90 gallon storage tank or in a food service quality 45 gallon wheeled trashcan (Note 5).
5. For animal storage, 6QT/5.7L Sterilite plastic boxes or comparable plastic/glass containers.
6. Animals can also be house in 5 gallon aquaria with minimal flow-through (Note 6).
7. For high animal volume labs, automated flow-through systems can be used to house large quantities of both juvenile (2-3L tanks) and adult (10L tanks) animals (Note 7).

Water temperature should be set to 18°C, pH 7.0, conductivity to 1500 μS/cm, with 5% daily water changes. The water flow in each tank should be kept low.

2.2 Raising axolotls from embryos to adulthood
1. Soda-lime glass bowls, 8 inch diameter.
2. Soda-lime glass bowls, 3.5 inch diameter.
3. 20% Holtfreter’s solution
4. Aeration bubblers
5. Soft mesh nets
6. Artemia brine shrimp and brine shrimp hatchery (Note 8).
7. California Blackworms (*Lumbriculus variegatus*) (Note 9).
8. Soft moist salmon pellets

### 2.3 Breeding

1. 28 quart plastic container (58.4 cm L x 41.3 cm W x 15.2 cm H) lined on the outside with foil.
2. Reusable ice packs
3. Broken terracotta pots or large flat natural rocks.
4. Soft mesh nets.

### 2.4 Disease treatment

1. 35 g/L salt solution
2. 5 mg/ml amikacin solution

### 2.5 Shipping

1. At least two water-sealed containers
2. Packing materials
3. Thick, sealable plastic bags
4. Disposable ice packs

### 3. Methods
3.1 Housing

1. Three times a week, change water in animal containers manually using a plastic colander or soft mesh net.
2. Scrub containers thoroughly with a brush at each water change (Note 10).
3. Feed animals on same schedule as water changes.
4. For aquaria, perform 10% water changes weekly.
4. Check pH, conductivity and water level of flow-through systems daily. Check ammonia levels every 2-4 weeks.
5. Monitor animal health daily.
6. For further sources on axolotl housing, see Note 11.

3.2 Raising axolotls from embryos to adults.

1. Place embryos in groups of 100 animals in aerated 20% Holtfreter solution (Note 12).
2. The expected death rate is 10%. Check for and remove dead embryos daily (Note 13).
3. Change water three times a week.
4. Embryos will hatch out of their eggs on their own and should be separated from unhatched animals at the next water change.
5. Larvae (Note 14) are housed in small groups of 20/container in 6QT plastic containers or 8 inch soda-lime glass bowls.
6. Feed larvae newly-hatched artemia brine shrimp daily.
7. Change water three times a week using a soft mesh net.
8. Split groups in half at the first sign of cannibalism and house animals of a similar size together.
9. Transition animals from brine shrimp to aquatic California Blackworms as soon as they
are large enough to eat the worms (approx. 3cm total length, Note 15).

10. At approximately 3cm SVL, juvenile (note 16) house at a density of 5 per 6QT container or 8 inch soda-lime glass bowl.

11. Transition animals to sinking soft moist salmon pellets at approximately 7 cm SVL.

12. Housing water is changed and animals are fed every Monday, Wednesday, and Friday.

13. House animals that are being used for limb regeneration experiments alone in a 3.5 inch soda-lime glass bowl (Note 17).

13. At approximately 5cm SVL, animals are transitioned to individual 6QT housing containers or into the automated flow-through systems in 3L housing containers.

14. Adult (Note 18) axolotls are either housed in individual 6QT containers or automated flow-through systems.

3.3 Breeding

1. Place one male and one female together in a 28-quart plastic container covered with aluminum foil. Include two reusable ice packs and a substrate of broken terracotta pots or large flat rocks (Note 19).

2. If spermataphores are observed in the tank after the first night, remove the male and add soft mesh nets to the container.

3. The female will lay eggs continuously over the next 12-24 hours, usually laying between 200 and 1000 eggs on the provided substrate.

4. The males can mate once a month and females at maximum every three months (Note 20).

3.4 Disease treatment

1. In order to prevent disease, keep animals well fed and in fresh water from the moment
eggs are laid to the death of the animal. Chronic stress is the most common cause of illness.

2. Catch signs of poor health early before disease spreads. This usually is apparent by a lack of appetite, loss of coloration in the gills, floating continuously, or loss of the “feathery” appearance of the gills.

3. A *columnaris* bacterial infection manifests as a white fungus-looking growth on the animal’s gills or flank.

4. Treat *columnaris*-infected animals with a high salt bath of 35g/L for 10 minutes for three consecutive days (Akimenko et al., 1995).

5. *Aeromonas* is a common parasite that manifests as red blotchy skin, often times on the animal’s leg.

6. Treat *Aeromonas* with three intraperitoneal injections of 5mg/ml amikacin solution at 5mg/kg body weight separated 48 hours apart.

7. For more information on axolotl diseases, please see (Avaron et al., 2006).

### 3.5 Shipping

1. Always ship axolotls with proper institutional animal care approval (Note 21).

2. Shipping should be performed in a water-sealed container such as a cooler or Styrofoam box inside a second cardboard box.

3. Place packing material within the interior container.

4. Place animals and water in a thick plastic bag, seal it, and place the bag within a second bag.

5. Seal bags with heat or securely tape them shut (Note 22).

6. Disposable ice packs should also be added to the shipping container.
7. Ship overnight only and remove animals from shipping containers to fresh water as soon as they arrive at their destination.

4. **Notes**

1. Restricting sources of natural light will increase the chances of successful matings taking place during the typically non-breeding months.

2. Water temperatures higher than 22°C are stressful on the animals and can have long-term negative effects on axolotl health.

3. A dimly-lit room is preferable to a bright one, as axolotls can be stressed by bright lights.

4. Success is also possible at a lower salinity of 750 μS/cm (Smith et al., 2008). Thus, axolotls thrive in a range of salinity conditions.

5. Free-standing water, especially tap water, should be treated with the water conditioners Amquel Plus and Novaqua Plus (Kordon LLC).

6. Aquarium filters should be used, but make sure that the water flow is kept to a minimum, as high flow is irritating to axolotls. Long-term housing in these tanks is reliable without any substrate, but small gravel can be used to act as a host for nitrifying bacteria and supplemented with 20% crushed coral to facilitate pH balance (Jessica Whited, pers. comm.).

7. The advantage of these systems is that the footprint is relatively and many of the daily tasks are automated and alarmed over network including water changes, water conductivity, pH, water flow, and temperature. Water-cooling is highly recommended with axolotls considering the heat that is generated by the automated systems. Most modern buildings can leverage process-cooling water, but if this is not available air-cooled units can be used for chilling as long as an exhaust vent for hot air is built into the animal room. The downside to an automated system is that the water is shared between tanks so disease could spread between animals. Therefore,
diligent observation of animal health is critical when using an automated system.

8. A simple brine shrimp hatchery can be generated using a 500ml glass bottle with a bubbler set at the bottom placed next to a window. Hatching solution should include 35g sea salt/L and 1.5g brine shrimp cysts per 500ml. Cysts will hatch after approximately 24 hours depending upon salinity, heat, light, and batch of artemia so optimization will be needed for each lab

9. A two-pound shipment of California Blackworms can be kept in the refrigerator in a large plastic container with just enough axolotl housing solution to cover the worms. Worms will survive for weeks if they are washed daily.

10. A small amount of sodium bicarbonate can assist in scrubbing.

11. Consult the AGSC before setting up an axolotl colony, read over their guide to axolotl care (www.ambystoma.org), and read the several reviews on raising and caring for axolotls in captivity (Martorana et al., 2001; Smith et al., 2008; Sugiyama et al., 2009; Voss et al., 2009).

12. Embryos left in their jelly coat will hatch in 2-3 weeks. The speed of development can be slowed if embryos are housed in colder temperatures

13. There is substantial variability in clutch-to-clutch embryo survival so close examination is needed to ensure maximal viability. It is important to remove dead eggs as they will foul the water and negatively affect the development of other embryos. Aerating the water with bubblers will increase animal survival and leads to faster embryo development.

14. Hatched axolotls are considered larvae until their hind limbs have developed to the digit stage.

15. We have found that feeding larval axolotls live blackworms promotes rapid growth.

16. Juveniles are categorized as animals that have completed development, but are not yet sexually mature. Animals range from approximately 3cm snout tip to vent (SVL) to 10-12cm
SVL. Axolotls do not undergo metamorphosis so it is not possible to determine a true transition from the larval to juvenile stage.

17. This ensures that limbs will be kept intact, as animals housed together may attempt to eat and injure each other.

18. Adults are categorized as animals that are sexually mature. Sexual maturity is highly variable in axolotls ranging from 8-15 months. For this reason, we categorize axolotls that are larger than 20cm snout tip to cloaca length as adults and greater than 10 months of age.

19. Two reusable ice packs are included to lower the temperature of the water to induce male mating behavior. On the first night of mating, males lay spermatophores onto the bottom of the tank and the female takes up one or more spermatophores into her cloaca. A substrate of broken terracotta pots or large flat natural rocks is added to provide substrate for the males to lay spermatophores.

20. We have found an approximate 30% success rate in the mating of healthy animals. Reproductive success is variable depending on the season and age of the animals. Matings are generally more successful during the winter and spring months, with lower success in the summer months.

21. Shipping axolotls is relatively straightforward and safe if the proper measures are taken to safeguard the welfare of the animal.

22. There is enough oxygen in the water for the animals to survive the trip.
**Figure 1.1.** Image of female leucistic (left), female albino (middle), and male wild-type (right) axolotls. Notice the large bellies of the two females indicating they are filled with eggs and the large cloaca of the mature male wild-type.
Figure 1.2. Cartoons of the exterior (top) and interior (bottom) of an adult leucistic axolotl. Each organ is drawn to approximate scale and represents the approximate position of each organ in the animal. Colors also represent the approximate color of each organ. The regenerating limb and tails represent mid-bud blastema stages. Internal histology of a mid-bud blastema is represented in Fig. 1.3C.
Figure 1.3. Histological images of axolotl tissues. A) Masson’s trichrome histological staining of a juvenile axolotl epidermis. Notice the large leydig cells (L) throughout the epidermis, dermal tissue underneath the epidermis (D), and muscle lying underneath the dermis (M). B) An transmission electron micrograph of a leydig cell in a juvenile axolotl epidermis. Notice the nucleus in the middle (N), which is surrounded by rough endoplasmic reticulum and a large number of dense vesicles (V). PM represents the leydig cell plasma membrane. K represents a keratinocyte with N representing the nucleus of the keratinocyte. C) Masson’s trichrome histological staining of a juvenile mid-bud limb blastema (BL). The bone is indicated with a B. Notice the thickened epidermis on the distal tip of the blastema and mesenchymal cell that makes up the blastema. D) Hematoxylin and eosin histological staining of a juvenile axolotl brain. The cross section is taken through the posterior telencephalon (forebrain; see Fig.1.2). Notice the ventricular zone that contain highly proliferative neural progenitor cells (Maden et al., 2013).
Chapter 2: Introduction to limb and organ regeneration

While the previous chapter introduced many aspects of the axolotl, including its ecology, anatomy, and husbandry, this chapter will introduce the regenerative abilities of the axolotl in greater depth. Axolotl limb regeneration is an unprecedented feat that marries both cellular proliferation and tissue patterning. While much of the molecular basis of this ability remains unknown, more than a century of research has laid the groundwork for contemporary studies of blastema formation and limb patterning. Although salamanders are most famous for their ability to regenerate limbs, their regeneration is not limited to appendages and in fact extends across many tissues and organs including the heart. Evidence is also mounting to suggest that the immune system plays a critical role in multiple examples of salamander tissue regeneration. The molecular biology of limb regeneration is thus a highly complex research question with implications that extend across tissues and systems.

This book chapter, published by Springer Press in the book *Regenerative Medicine- from Protocol to Patient* (Farkas et al., 2016a) was a collaborative effort between myself and several other members of the lab. It describes various modes of salamander regeneration across organs and tissues, and concludes with a table summarizing all research of salamander regeneration across organs and tissues. It also provides a contemporary overview of the current state of molecular biological research in salamander regeneration, as well as an introduction to the role of the immune system, which will be further elaborated on in Chapter 5. I am listed as first author because I both contributed the greatest amount of content and because I was the lead editor in charge of integrating all of the disparate parts together to make the manuscript read more smoothly. The chapter has been edited to trim those aspects which are less-pertinent to my work, and the parts that remain are predominantly constituted of my own writing.
Organ and appendage regeneration in the axolotl

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Introduction

Regeneration is a remarkable feat of biology. It requires an organ system – most often consisting of many different cell types - to stop its specialized function and step back in ontological time. Regeneration overcomes the general understanding that development is a one-way street. We now know that there is great variability in regeneration capacity across phylogeny, likely because animals need to have some mechanism in place to survive injuries or diseases that it will encounter. Some animals, including humans, meet this need by closing the wound as quickly as possible and making due with the deficit. Other animals, such as the axolotl described here, instead regenerate the damaged or missing tissue.

*Ambystoma mexicanum*, commonly known as the axolotl, is uncommon among vertebrates because of its superior regenerative abilities. References of their unique abilities cross from the scientific to the mainstream media, but they are also useful animals for understanding the mechanisms that regulate regeneration. As axolotls are tetrapods that can breed all year-round and accept grafts between adults, they possess some major advantages that make them uniquely suited to be animal models of regeneration. In terms of appendage regeneration, there is currently a lack of animal models for complex regeneration. The two dominant models are zebrafish, which regenerate caudal fins throughout life, and *Xenopus* frogs, which can regenerate limbs and tails early in development. Although these systems have provided important insights into vertebrate regeneration, the axolotl limb is particularly well suited to study adult appendage regeneration. Axolotls are capable of regenerating complete
adult limbs that are morphologically similar to human limbs using endochondral ossification. In contrast, the zebrafish dermal caudal fin skeleton has no mammalian counterpart, and the fin skeleton regenerates by direct ossification from mature osteocytes (Knopf et al., 2011; Sousa et al., 2011). *Xenopus* regenerates early in development, but its ability to regenerate patterned skeletal structures is absent in adulthood. Therefore, the axolotl is the best vertebrate model for adult joint regeneration.

Although axolotls have been studied for almost 200 years, only recently have technological advances helped revive the axolotl into a model organism in modern regeneration biology (Voss et al., 2009). The axolotl is becoming a chosen model for regenerative biology because it can regenerate more completely than any other vertebrate including the brain, spinal cord, heart, limb, tail, and portions of the eye. Modern genomic tools are available including microarray analysis (Campbell et al., 2011; Monaghan et al., 2012; Monaghan et al., 2009; Monaghan et al., 2007), RNAseq (Knapp et al., 2013; Monaghan et al., 2009; Stewart et al., 2013), a genomic map (Smith et al., 2005a), genomic sequence data (Smith et al., 2009), an active genome sequencing project, and bioinformatic databases (Smith et al., 2005b). Functional testing of genes is also available through the generation of transgenics (Khattak et al., 2014; Monaghan & Maden, 2012b; Sobkow et al., 2006a; Whited et al., 2012), knock-down of genes by morpholinos (Schnapp et al., 2005; Zhu et al., 2012), over-expression of genes by electroporation (Mercader et al., 2005) and viruses (Khattak et al., 2013; Whited et al., 2013), and cell tracking by tissue grafting between GFP and white axolotls (Nacu et al., 2009). With this array of modern tools, married with the qualities that have made the axolotl a subject of research for hundreds of years, the axolotl system has become a powerful model to dissect the mechanisms that regulate development and regeneration.

Here, we will highlight what is known about the axolotls’ regenerative abilities and discuss
the mechanisms that regulate regeneration each organ system. It is generally assumed that the axolotl has the ability to regenerate most if not all of its tissues, but a survey of tissue regeneration has yet to be performed in this animal model. We will focus upon the regenerative capacity of the axolotl, but it is necessary to include examples of regeneration in the newt, *Xenopus laevis*, and zebrafish because in some aspect these species have been studied in more detail than in the axolotl model.

**Axolotl limb regeneration**

Though the axolotl possesses many extraordinary regenerative capabilities, its ability to fully regenerate amputated limbs is among its most striking and well-studied. Neotenic urodele salamanders are the only vertebrates capable of regenerating limbs throughout adulthood, and thus this process has been the subject of scientific study and fascination for more than two centuries (Spallanzani, 1769). Though many of the molecular mechanisms underlying limb regeneration remain poorly-understood, past studies have nevertheless elucidated the major steps and some underlying mechanisms of the process.

Axolotl limb regeneration occurs in a series of stages that are morphologically and transcriptionally distinct (Voss et al., 2015). Amputation of the limb induces rapid vasoconstriction, which serves to minimize blood loss from the injury. Clotting is very rapid, as is epidermal migration and closure of the wound site. So long as excess bone is trimmed and kept from protruding from the wound site, wound closure will occur within 24 hours post-amputation. Over the next several days, the wound epithelium thickens and forms a structure called the apical epithelial cap (AEC), which intimately contacts the mesenchyme in the absence of the dermis. This AEC is a crucial component of limb regeneration- if it is prevented from forming or replaced with fully-thickened epidermis, regeneration does not take place (Loyd &
Tassava, 1980; Tassava & Garling, 1979). It is believed that the AEC secretes a host of factors, including metalloproteases, which are necessary for breaking down the extracellular matrix (ECM) of the underlying tissue, thus permitting and guiding cell migration and accumulation in the mesenchyme (Lévesque et al., 2007; Thornton, 1960a; Yang & Bryant, 1994; Yang et al., 1999). Once a critical mass of dedifferentiated cells accumulates below the wound epithelium, the next stage of regeneration is initiated and a proliferative mass called the blastema is formed. Although the overall rate of limb regeneration largely depends on the size of the animal, the switch from wound healing and cell accumulation to blastema formation generally occurs at around ten days post-amputation (DPA). Once the blastema has formed, cell cycling and proliferation increases dramatically (Loyd & Tassava, 1980) as blastemal cells divide rapidly and promote the outward growth of the regenerating structure.

**Characteristics of the blastema**

Though it appears to be a homogeneous population of near-identical cells, the blastema is in fact comprised of local (within 1-2mm of the wound site (Butler, 1935; Butler & O'Brien, 1942)) dedifferentiated cells arising from several different tissue types, including fibroblasts, Schwann cells, and satellite cells (Monaghan & Maden, 2012a). This last cell type represents a crucial distinction between axolotls, which are members of the family Ambystomatidae, and newts, which are members of the family Salamandridae. Though newts and axolotls bear superficial similarities and are both studied for their regenerative capabilities, the families in fact diverged 145 million years ago (Zhang & Wake, 2009) and certain differences have been noted in their regenerative mechanisms. Of note is the fact that while satellite cells dedifferentiate and contribute to the blastema in the axolotl, in newts it appears that myocytes themselves dedifferentiate (Sandoval-Guzman et al., 2014). Caution is thus advised when applying findings
from the newt to the axolotl, and vice versa. However, one feature that remains constant across both examples of limb regeneration is the fact that all blastemal cells are lineage-restricted: that is, once regeneration is nearly complete, they re-differentiate back into their tissues of origin. Thus, satellite cells eventually differentiate into myocytes while Schwann cells will only become Schwann cells. The lone exception is that of fibroblasts, which can differentiate into either fibroblasts or chondrocytes (Kragl et al., 2009). Dedifferentiated fibroblasts make up a disproportionately large percentage of the regenerating blastema (Muneoka et al., 1986), and their plasticity allows the limb to fully regenerate even if all skeletal elements are removed prior to amputation (Foret, 1970; Thornton, 1938). Although the mechanisms behind this cellular “memory” remain unknown, one can safely qualify the blastema as a collection of generally lineage-restricted, dedifferentiated cells arising from multiple tissue types.

**Nerve dependence and molecular mechanisms of limb regeneration**

One curious characteristic of blastemal formation and growth is the fact that it is nerve-dependent. If the limb is denervated prior to or shortly after amputation via simple transection of the brachial nerves, the wound heals over without incident but formation of the blastema does not occur and regeneration does not go forward. This phenomenon has been the source of scientific curiosity and study since it was first discovered in the early 1800’s (Todd, 1823), and nerve dependence is in fact found in numerous examples of regeneration and wound healing across phylogeny. From starfish arm regeneration to mammalian ear punch healing, an intact nerve source appears to be imperative for proper regenerative growth (Kumar & Brockes, 2012). Though the molecular mechanisms underlying this nerve dependence remain poorly-understood in the axolotl, a host of studies spanning the past half-century have done much to elucidate the roles of these nerves during limb regeneration.
Nerves heavily invade the wound epithelium within several days after amputation, and they appear to be critical for the maintenance of the AEC, as early denervation eventually results in the collapse of the AEC and the impairment of blastema formation. Nerves further appear to be necessary for the maintenance of blastemal proliferation. Denervation once the blastema has formed starkly reduces the proliferation of blastemal cells (Goldhamer & Tassava, 1987; Maden, 1978a; Tassava et al., 1974), though it does not appear to affect cell differentiation or limb patterning. Thus, denervation well after blastema formation (at around 15 DPA) actually induces the formation of a miniature, fully-patterned limb (Powell, 1969; Schotté & Butler, 1944; Singer & Craven, 1948). Axolotl peripheral nerves are themselves capable of regeneration and will in fact rapidly regrow after denervation. Consequently, studies involving limb denervation must be careful to re-denervate every 7-10 days and cannot last longer than approximately 20 days, after which re-denervation is effectively impossible and regeneration of the limb goes forward. This characteristic represents another divergence between newts and axolotls, as amputated newt limbs do not recover from brachial nerve transection and will not regenerate even up to 72 days post-denervation (Liversage & McLaughlin, 1983).

A series of elegant experiments performed by Marcus Singer in the 1950’s characterized the nature of nerve dependence in the regenerating axolotl limb. Singer found that a certain number of nerve fibers is necessary for regeneration, although this amount varies depending on the site of amputation- if the number of nerves present falls below a specific threshold, regeneration does not take place. Singer also found that the critical function of the nerves does not involve action potentials or neurotransmitter release, nor does it require sensory or motor neuron innervation in particular. Instead, nerve support of the wound epithelium and blastema appears to be trophic in nature (Singer, 1952a, 1964). These experiments suggest that nerves-
and the dorsal root ganglia, implantations of which are capable of rescuing regeneration in
denervated limbs (Goldhamer et al., 1992; Kamrin & Singer, 1959; Tomlinson & Tassava,
1987)–release factors which are critical for inducing blastemal formation and maintaining
blastemal proliferation. The precise identities of these factors remain largely unknown, although
evidence has been gathered in support of many–including neuregulin-1 (Wang et al., 2000a),
transferrin (Kiffmeyer et al., 1991; Mescher et al., 1997), fibroblast growth factors (Satoh et al.,
2011), and anterior gradient protein (Kumar et al., 2007b). Identification of these critical nerve-
derived factors thus constitutes a major topic of regenerative science moving forward.

The early invasion of macrophages into the wound site is also critical for limb
regeneration. Total macrophage ablation prevents regeneration and induces aberrant fibrotic
deposition in the wound site (Godwin et al., 2013). Limb regeneration like all forms of axolotl
regeneration is a totally scar-free process that occurs with minimal collagen deposition
(Levesque et al., 2010; Seifert et al., 2012c). Instead of collagen, a dynamic network of
fibronectin provides loose structure for the regenerating blastema (Maden & Keeble, 1987; Rao
et al., 2009). It is therefore possible that macrophages, which peak in number at approximately 4
DPA (Godwin et al., 2013), are necessary for maintaining a permissive regenerating environment
erly after injury. Further studies have also demonstrated that macrophages play a role in
clearing senescent cells from the wound site and blastema during regeneration (Yun et al., 2015).
It is thus believed that macrophages have multiple crucial functions in axolotl limb regeneration,
and the study of these functions remains an ongoing process.

**Limb patterning and mammalian appendage regeneration**

One extraordinary characteristic of axolotl limb regeneration is the fact that perfect
regeneration occurs regardless of the site of amputation. Thus, amputation at the shoulder will
result in regeneration of the entire limb, while amputation distal to the elbow joint will regenerate only distal tissues. Considerable research has therefore been devoted to elucidating the molecular underpinnings of axolotl limb patterning. Studies have found that fibroblast-derived blastemal cells demonstrate a “memory” of their initial position (Kragl et al., 2009), and this position is in some way expressed on the cell surface, as proximal cells cultured in vitro reliably engulf distal cells (Nardi & Stocum, 1984). These cells are thus capable of detecting discrepancies in the proximal/distal and dorsal/ventral axes and subsequently intercalating any missing structures, though the stability of this positional memory varies and can be reprogrammed in early and distal blastemal cells if they come into contact with more stable proximal cells (McCusker & Gardiner, 2013). A host of studies have demonstrated that retinoic acid (RA) plays crucial roles during limb patterning. Application of exogenous RA early after amputation induces its expression in (Monaghan & Maden, 2012b) and proximalizes (Keeble & Maden, 1989; Niazi et al., 1985) fibroblast-derived blastemal cells, completely erasing distal positional memory and suggesting that positional cell memory is in some way maintained via a proximodistal retinoic acid gradient throughout the limb (Scadding & Maden, 1994).

Meanwhile, the salamander-specific cell surface protein Prod1, which is upregulated in the blastema in response to RA (da Silva et al., 2002), may mediate cell adhesion differences. Prod1 ablation impairs proximal cell engulfment (da Silva et al., 2002) and overexpression of the protein proximalizes distal blastemal cells (Echeverri & Tanaka, 2005). The molecular mechanisms of limb patterning remain under investigation, and as salamander limb patterning is itself a topic vast enough to fill an entire chapter, numerous reviews have covered the process in greater detail (Mariani, 2010; McCusker et al., 2015; McCusker & Gardiner, 2014; Stocum & Cameron, 2011).
The axolotl’s extraordinary ability to fully regenerate amputated limbs sets it apart from mammals and virtually all other vertebrates outside the urodeles, but some regenerative mechanisms are conserved across phylogeny. Mammalian appendage regeneration is very limited in scope—mice can regenerate only the tips of their digits (Borgens, 1982) while human children are capable of regenerating amputated fingertips so long as the wound is not immediately sealed after injury (Illingworth, 1974). Like axolotl limb regeneration, mammalian digit tip regeneration relies on a heterogeneous mix of lineage-restricted progenitor cells (Lehoczky et al., 2011) and is disrupted if there is no intact nerve source, although nerve dependency in this case appears to affect tissue patterning more than cell proliferation (Rinkevich et al., 2014). However, nerve dependency during mouse ear hole punch regeneration appears to align closely with salamander nerve dependency, as denervation of the ear prevents blastema formation and induces necrosis (Buckley et al., 2012). This overlap further underlined by the conserved presence of msx1, a homeobox-containing gene that is highly upregulated during both mammalian digit tip (Allan et al., 2006; Reginelli et al., 1995) and axolotl limb regeneration (Koshiba et al., 1998). Axolotl limb regeneration thus shares some essential similarities with mammalian digit tip regeneration, and consequently it offers an enticing model for the study and advancement of regenerative medicine in mammals.

**Cardiac regeneration**

Regeneration of the heart myocardium is observed to some extent in most vertebrates including zebrafish (Poss et al., 2002b), mice (Porrello et al., 2011), frogs (Rumyantsev, 1966), and newts (Oberpriller & Oberpriller, 1974). It is commonly believed that lower vertebrates can recover function throughout life while mice can only regenerate up to 7 days after birth (Porrello et al., 2011). Although only three studies have experimentally demonstrated myocardial
regeneration in the axolotl, the evidence supports that axolotls can regenerate adult myocardial tissue. Regeneration of the cardiac tissue after 10-15% partial ventricular amputation is demonstrated in all three of these studies (Cano-Martinez et al., 2010; Flink, 2002; Vargas-Gonzalez et al., 2005). In the axolotl, the completeness of regeneration has not been assessed yet. In newts, it is known that cardiac regeneration occurs without any presence of a scar (Witman et al., 2011). Continuous BrdU labeling from 14-21 days post injury in axolotls showed that the majority of BrdU+ cells were found within 75-125 µm of the injury site. In addition, co-staining with cardiomyocyte-specific antibodies showed that 74.3% of epicardial cells and 12.8% of cardiomyocytes were BrdU+. Some BrdU+ cells were found as far as 750 µm away from the injury site suggesting a widespread proliferative response after cardiac injury (Flink, 2002). Most functional recovery occurs within 30-90 days post injury, which is preceded by cardiomyocyte proliferation (Cano-Martinez et al., 2010). Co-labeling of proliferative markers with cardiomyocyte markers suggest that cardiomyocyte dedifferentiation drives the regenerative process (Cano-Martinez et al., 2010; Flink, 2002; Vargas-Gonzalez et al., 2005), which is supported by the fact that adult ventricular cardiomyocytes in newts can readily proliferate in vitro (Mercer et al., 2013; Nag et al., 1979; Tate et al., 1989). Furthermore, genes involved in the embryonic cardiogenic programming including Hand2, Nkx.2, Gata4, Islet1, and Gata5 are all upregulated during newt cardiac regeneration with a significant proportion of Gata4 and Islet1 expression co-localizing with cardiomyocyte markers supporting the likelihood of cardiomyocyte dedifferentiation (Witman et al., 2011).

Based on the evidence, it is likely that axolotls regenerate both by a local injury response (epimorphic) and a widespread organ-wide (compensatory) mechanism. A similar mechanism of cardiomyocyte proliferation was observed in endogenous cardiomyocytes during zebrafish
regeneration after ventricular cryoinjury (Sallin et al., 2015). Moreover, cellular lineage tracing in zebrafish have shown that dedifferentiation of resident cardiomyocytes generate the new myocardium in zebrafish (Jopling et al., 2010; Kikuchi et al., 2010; Zhang et al., 2013), which also seems to be the case in the regenerating neonatal mouse (Mahmoud et al., 2013; Porrello et al., 2011; Porrello et al., 2013). Although a more comprehensive analysis of heart regeneration is required in amphibians to elucidate their mechanism of regeneration, it is intriguing to think that all animals with the capability to regenerate heart myocardium do so using the same basic mechanisms.

**Early wound healing and the role of the immune system**

Regeneration across all tissue types is a complex process dependent on inflammation, tissue remodeling, and tissue formation. Blood and its circulation plays a major role in orchestrating these events by closing off the initial wound and carrying necessary factors to the wound site. Here, we will cover what is known about the axolotl’s clotting factors and the immune system’s role in regeneration, with a particular emphasis on the role of leukocytes for this process.

**Clotting factors**

An early step in regeneration is the formation of a clot mediated by cleavage of plasma fibrinogen by thrombin. The clot not only closes off the wound to prevent infection, but may also provide activating factors required for regeneration. Axolotl blood clots within several minutes after amputation, and the wound epidermis surrounds the clot within 12-24 hours depending upon the size of the wound (Sobkow et al., 2006a). It is possible that clotting factors released early after injury may be an inductive signal. Indeed, a thrombin-generated ligand is known to induce newt myotubes to re-enter the cell cycle in culture (Tanaka et al., 1997), supporting the
hypothesis that thrombin is required for regeneration. In the regenerating newt lens, thrombin activity is present and required for regeneration (Imokawa & Brockes, 2003). Thrombin seems to be associated with regenerating tissues, but it is not known whether it is sufficient to induce a regenerative response. Furthermore, the thrombin-mediated ligand or its downstream targets have not been identified, making it difficult to know what role it plays during regeneration. Regardless, clotting is associated with the early injury response and therefore is a prime candidate for inducing a regenerative response.

**Inflammation and the immune system**

The axolotl immune system is comprised of an innate immune system and a rudimentary adaptive immune system. Axolotls are deemed relatively immunodeficient due to the fact that they only produce two immunoglobulin classes (IgM and IgY) - neither of which are anamnestic - and overall humoral and cytotoxic responses are slow or non-existent (Chen & Robert, 2011; Godwin & Rosenthal, 2014; Tournefier et al., 1988). IgM is produced by lymphocytes within the spleen around 7 weeks post-fertilization, though IgY is not detected until the axolotl reaches 7 months old (Fellah et al., 1989). It has been surmised that inflammation and immunomodulation may be implicated in regenerative ability because there is an inverse relationship between the maturation of the immune system and capacity to regenerate (Harty et al., 2003; King et al., 2012; Mescher & Neff, 2005). Inflammation is an initial response to wounding. Upon injury to mammalian tissue, cytokines direct immune cells to the area of the assault, thus creating inflammation and inducing scar formation (Wynn, 2008). Contrastingly, the larval axolotl lacks neutrophils and macrophages that migrate to the wound bed, and the adult axolotl has a low number of neutrophils found in the wound bed (Levesque et al., 2010; Seifert et al., 2012b;
This reduced inflammatory response correlates with scar-free wound healing of the axolotl (Seifert et al., 2012b), which is reminiscent of the scar-free wound healing capabilities of fetal mammals (Namazi et al., 2011). This is supported by studies which have shown that after 24 weeks of gestation, onset of a high inflammatory response is consistent with low regenerative capacity and scar formation in mammals (Adzick & Lorenz, 1994; Xue & Jackson, 2015; Yates et al., 2012). Despite this clear inverse correlation, the molecular mechanisms behind how the immune cells affect regeneration is poorly understood.

Macrophages specifically seem to be important in the regeneration process. Their roles are unclear, but they likely contribute through the phagocytosis of debris following initial injury, breakdown of extracellular matrix (ECM) and promotion of its reconstruction, release of pro- and subsequently anti-inflammatory cytokines, and mobilization of stem cells. Depletion of macrophages in the axolotl limb immediately after injury prevents blastema formation, and depletion of baseline-level macrophages 15 days post injury will prolong total regeneration time (Godwin et al., 2013). In mouse bone marrow studies, macrophage depletion caused hematopoietic stem cells to egress from the niche due to a loss of paracrine homeostasis factors such as Cxcl12 (Chow et al., 2011). In zebrafish, depletion of macrophages also leads to defective caudal fin regeneration (Li et al., 2012). Macrophage depletion has also been implicated in prevention of heart regeneration in the adult axolotl, though if they are required for merely debris clearance, cytokine signaling, or a greater paracrine role is still not clear (Pinto et al., 2014). Overall, the ability of macrophages to alter their environment physically and through molecular signaling are clearly essential to tissue regeneration, though the extent of these mechanisms are not yet fully understood. While the immune system is implicated in many aspects of regeneration, the entire blood lineage itself shows a high level of regenerative capacity.
in the axolotl. Ablation of the liver or spleen by targeted irradiation of the adult axolotl will lead to anemia and eventually death (Lopez et al., 2014). Furthermore, the liver and spleen may provide different niches as the HSPC populations of the spleen are 1000-fold enriched in lymphoblastic populations compared to the periphery of the liver (Lopez & E.W., 2015).

**Conclusion**

Based upon the examples in this review, axolotls utilize multiple mechanisms to coordinate a regenerative response, but it is clear that with time the animal will fully regenerate its limb, tail, spinal cord, and heart. In all injuries models studied, injury initiates a local infiltration of leukocytes, which is associated with migration of cells nearby the injury. Migration of cells is followed with a sustained proliferation of progenitor cells that arise either from dedifferentiation or recruitment of local adult stem cells, most commonly located near the injury site. Differentiation of progenitor cells is a recapitulation of development of each organ, coupled with enhanced growth until the injured tissue is replaced. The molecular processes that initiate a regenerative response or regulate when an organ stops growing are critical questions that need to be elucidated to in order to understand the regenerative process.

The axolotl’s regenerative abilities are not only limited to brain, spinal cord, nerve, tail, heart, limb and skin regeneration. The regenerative ability of other organs are highlighted in Table 2.1. Considering the broad regenerative ability that has been described here, it is likely that most if not all organs and tissues can regenerate at some point in their lifetime. For example, it was previously thought that axolotls could not regenerate their lens. This idea was recently overturned, showing that for a duration of 2 weeks after hatching/Stage 44, axolotls possess the ability to regenerate lens from the ventral or the dorsal iris or simultaneously from both
(Suetsugu-Maki et al., 2012). Overall, given its wide variety of organ and appendage regenerating capabilities the axolotl is a compelling model for the study of adult tissue regeneration. A broad survey of organ regeneration should provide much insight into the common and divergent mechanisms that regulate regeneration.

<table>
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<tr>
<th>Tissue</th>
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<tr>
<td>Retina</td>
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<td></td>
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<td>(Akimenko et al., 2003; Jiang et al., 2016; Minelli &amp; Del Grande, 1974)</td>
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<td>newts – yes</td>
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<tr>
<td>Spinal cord</td>
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<td>(Butler &amp; Ward, 1965, 1967; Egar &amp; Singer, 1972; McHedlishvili et al., 2012; Piatt, 1955)</td>
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<td></td>
<td>newts – yes</td>
<td></td>
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<tr>
<td>Lens</td>
<td>Ambystoma– up to two weeks post hatching newts – yes</td>
<td>(Collucci, 1891; Eguchi et al., 2011; Henry &amp; Tsonis, 2010; Sousounis et al., 2013; Suetsugu-Maki et al., 2012; Wassmer et al., 2013)</td>
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<td>Jaw &amp; teeth</td>
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<td>(Ghosh et al., 1994; Goss &amp; Stagg, 1958; Graver, 1974; Spallanzani, 1769)</td>
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<td></td>
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<td></td>
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<td>Lungs</td>
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<tr>
<td>Liver</td>
<td>Ambystoma - yes</td>
<td>(Heberlein, 1930; Williams, 1961)</td>
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<tr>
<td></td>
<td>newts - yes</td>
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<td>(Garavini, 1977)</td>
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<td>N/A</td>
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<td>Lateral line</td>
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<tr>
<td>Tail</td>
<td>Ambystoma - yes newts - yes</td>
<td>(Holtzer, 1956; Iten &amp; Bryant, 1976)</td>
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</table>

Table 2.1. The earliest citation that clearly describes the regenerative ability of each animal group. Newts is represented by *Cynops pyrrhogaster, Triturus cristatus, Pleurodeles waltl, and Notophalamus viridescense*. Ambystoma represents either *Ambystoma maculatum* or *Ambystoma mexicanum* (axolotl).
Chapter 3: Introduction to Nerve Dependent Regeneration

While the previous two chapters sought to introduce the axolotl and its considerable regenerative abilities, the ultimate goal of my research is to determine the molecular basis for the nerve dependence of axolotl limb regeneration. Nerve dependence, as briefly defined in the previous two chapters, is the absolute requirement of peripheral nerves for limb regeneration. If the axolotl limb is denervated via surgical severance of the peripheral nerves at the brachial plexus, regeneration of the limb is completely blocked. While nerve dependent regeneration was first discovered nearly two centuries ago, much remains to be learned about the molecular mechanisms that drive this phenomenon. Although humans are of course comparatively poor regenerators, the study of nerve dependence harbors major implications for human health, as peripheral nerves are critical for human wound healing and peripheral nerve damage is a leading cause of non-traumatic amputations. Uncovering the role of nerves during nerve dependent axolotl regeneration therefore has multiple implications: it elucidates the cell signals which promote complex salamander tissue regeneration while also informing future studies of the molecular mechanisms of nerve dependent wound healing in other organisms.

The following manuscript is a recently accepted review that covers the history of nerve dependence research in the axolotl and beyond. This review was solicited by the journal *Neurogenesis* in recognition of our axolotl findings described in Chapter 4. However, it provides a thorough introduction as well as a contextual basis for the published work that will be described in the next chapter. Our goal was to provide a brief history of the discovery and study of nerve dependence, along with an appreciation of its ubiquity among vertebrates and a contemporary overview of the current state of the field. This manuscript was recently accepted
A Brief History of the Study of Nerve Dependent Regeneration
Farkas, Johanna E. & Monaghan, James R.

Abstract
Nerve dependence is a phenomenon observed across a stunning array of species and tissues. From zebrafish to fetal mice to humans, research across various animal models has shown that nerves are critical for the support of tissue repair and regeneration. Although the study of this phenomenon has persisted for centuries, largely through research conducted in salamanders, the cellular and molecular mechanisms of nerve dependence remain poorly-understood. Here we highlight the near-ubiquity and clinical relevance of vertebrate nerve dependence while providing a timeline of its study and an overview of recent advancements towards understanding the mechanisms behind this process. In presenting a brief history of the research of nerve dependence, we provide both historical and modern context to our recent work on nerve dependent limb regeneration in the Mexican axolotl.

Introduction
Tissue regeneration has been a source of scientific study and fascination for nearly five hundred years, but the mystery of how and why certain organisms regenerate lost tissue remains as enigmatic and compelling as ever. One major question which has emerged throughout the past centuries of research is that of nerve dependent regeneration. Nearly every known example of vertebrate tissue regeneration requires the presence of intact peripheral nerves, a striking commonality given the vast diversity of regenerating organisms and tissues. Although nerve
dependence was first discovered in the salamander nearly two hundred years ago, the molecular mechanisms underpinning this phenomenon have only recently begun to come to light.

**Nerve dependence across models of vertebrate tissue regeneration**

Vertebrates display a vast range of regenerative abilities, and nerve dependence has been observed in regenerative species ranging from fish to mammals. Zebrafish cannot regenerate amputated fins if they have undergone denervation (Simões et al., 2014), and nerve dependence has also been observed in the regenerating barbels of catfish (Goss, 1956a). African clawed froglets (*Xenopus laevis*) generate a nerve dependent hypomorphic spike after amputation, whereas hindlimb regeneration in larval frogs becomes progressively more nerve dependent as the animal develops (Filoni et al., 1995; Suzuki et al., 2005). This progression is likely due to the abundance of mitogens during early limb development (Filoni et al., 1999).

Anuran amphibians appear to forego their ability to regenerate complete appendages after metamorphosis, but urodele amphibians demonstrate nerve dependent limb regeneration throughout adulthood. A variety of newt and ambystomatid salamander species, among which the Mexican axolotl (*Ambystoma mexicanum*) is the most widely studied, have been used as models for regeneration research. Urodele limbs are anatomically similar to human limbs and are generally capable of regenerating after amputation regardless of the age of the animal. This regeneration normally proceeds via the formation and subsequent growth of a hyperinnervated proliferative mass called the blastema. However, denervation of the salamander limb completely prevents the formation of this blastema and halts regeneration early in the process. The salamander is thus a robust and reliable model for the study of nerve dependence, and it will
therefore be the main focus of this review. For further review of known examples of nerve
dependence in both vertebrates and invertebrates, see (Kumar & Brockes, 2012).

Mammals are not generally known for their regenerative prowess, but nerves do appear to
play an important role during both early and adult mammalian tissue regeneration. Fetal
mammals are capable of scarless epidermal wound regeneration, but denervation disrupts this
regenerative program in both mice (Kishi et al., 2006) and lambs (Stelnicki et al., 2000).
Prenatal nerve dependence is not limited to mammals, as wound healing in the developing chick
embryo is also dependent on cutaneous innervation (Harsum et al., 2001). Furthermore,
denervation of the vagus nerve inhibits cardiac tissue regeneration in the neonatal mouse and can
be rescued via supplementation with nerve growth factor and Neuregulin-1 (Mahmoud et al.,
2015).

Although adult mammals are far more limited in their regenerative abilities, mice are
capable of regenerating their digit tips so long as the amputation plane does not extend past the
nail bed. Few studies have looked at innervation during this process, and thus the role and
necessity of nerves for the support of mammalian digit regeneration remains unclear. In 2014,
Rinkevich et al. found that denervation of the amputated digit tip led to defects in digit
patterning, although cell turnover was unaffected (Rinkevich et al., 2014). In contrast, a 2016
study by Johnston et al. indicated that Schwann cells dedifferentiate after amputation and secrete
essential paracrine factors, including oncostatin M and PDGF-AA, that act to stimulate
mesenchymal cell proliferation (Johnston et al., 2016). Study of the nerve dependency of ear
hole-punch regeneration also remains in its early stages. Denervation leads to tissue regression
and necrosis in mouse strains which are capable of healing ear wounds (Buckley et al., 2011).
Ear tissue regeneration in the African spiny mouse proceeds from the innervated (proximal) side
of the wound, and the regenerating tissue is densely innervated by invading peripheral nerves (Gawriluk et al., 2016; Seifert et al., 2012a). While much remains to be learned about the role of nerves during mammalian tissue regeneration, it is clear that nerve dependence represents an exciting avenue for further research in both fetal and adult animal models.

Although humans display relatively little capacity for tissue regeneration, nerves nevertheless play an important role in human wound healing, and loss of innervation is a leading cause of chronic wound formation. Paraplegic and quadriplegic patients experience more complications and develop more chronic wounds than nonparalegic patients during wound healing in their denervated regions (Basson & Burney, 1982). Moreover, peripheral neuropathy in diabetic patients can result in impaired wound healing and the formation of diabetic ulcers on the extremities, a condition which has become one of the leading causes of non-traumatic lower limb amputations in developed countries (Johannesson et al., 2009; Organization, 2016). This pathology does not appear to be a direct result of insulin resistance or depletion, but is instead partially caused by aberrant apoptosis and inflammation in denervated skin wounds (Brown et al., 1997). For further review of the role of nerves during mammalian wound healing, see (Barker et al., 2006). Elucidating the underlying mechanisms of nerve dependency thus has wide-ranging implications for the study of human health and medicine.

**History of nerve dependence research in the salamander**

The study of nerve dependency began nearly two centuries ago, and salamanders have been the subject of this research from the very beginning. For a brief timeline of this history of study, see Figure 1. Salamanders have proven an ideal model for the study of nerve dependency because their brachial nerves are easy to surgically access and denervation of the amputated salamander limb reliably inhibits regeneration. An anonymous aquatic salamander featured in
what is credited to be the first study demonstrating the requirement of nerve axons for regeneration. In 1823, Tweedy John Todd submitted a comprehensive overview of salamander regenerative capacity to the Quarterly Journal of Science, Literatures and the Arts. In it, he noted for the first time that:

“If the sciatic nerve be intersected at the time of amputation, that part of the stump below the section of the nerve mortifies... If the division of the nerve be made after the healing of the stump, [regeneration] is either retarded or entirely prevented. And if the nerve be divided after [regeneration] has commenced, or considerably advanced, the new growth either remains stationary, or it wastes...” (p.91, (Todd, 1823)).

Despite the novelty of these findings, which accurately depicted the results of denervation at various stages during regeneration, Todd’s remarkable observations were left largely untouched for more than seventy years.

As the 19th century came to a close, a number of researchers independently attempted to repeat Todd’s experiments, and in doing so led to a renewed interest in nerve dependent limb regeneration. Because the regenerating salamander provided an accessible and re-usable model for the study of tissue development, the basis for many of these early studies arose from the desire to study the role of nerves on tissue growth, rather than direct interest in the phenomenon of regeneration. In 1913, the Scottish physician Diarmid Paton wrote an extensive contemporary overview of turn-of-the-century nerve dependence studies (Noël Paton, 1913). In his series of lectures, entitled “The Nervous and Chemical Regulators of Metabolism,” Paton describes a research environment characterized by equal parts enthusiasm and controversy. Although
several scientists, led by German researchers Wolff (Wolff, 1895) and Walter (Walter, 1911), reported that they had reproduced Todd’s experiment, the wide variety of salamander species available to study led to a heated debate on the ubiquity of amphibian nerve dependency. Paton, himself convinced by Wolff and Walter’s work, rather scathingly writes that one scientist, attempting to reject the nerve dependence hypothesis, decided to study the newt *Triturus viridescens* “after rejecting various amphibia because the results were not satisfactory” (p.13). Nevertheless, it appears that the scientific community was united enough at this point in time for Paton to confidently declare “with the large series of experimental investigations upon [nerve dependence], we need not concern ourselves further than we have already done” (p.14). His question for salamander researchers going forward, “to what extent does the nervous system dominate the metabolism?” (p.14) has resonated throughout the twentieth century and up to the modern day.

This increased focus on the presence and necessity of nerves led salamander researchers to concentrate on parsing out the contributions of various types of nerves. While debate over the contributions of different types of nerves persisted for decades, the lab of Marcus Singer put many of these arguments to rest throughout the middle part of the 20th century. In a series of seminal works published during the 1940’s and throughout the following decades, Singer found that the type of nerve present has no effect: instead, all nerve fibers contribute roughly equally to the process and nerves must be present in sufficient quantity to trigger regeneration ((Singer, 1946; Singer, 1952a; Singer & Craven, 1948), for review of Singer’s work see (Singer, 1964; Singer, 1978)). While the precise cause of this “nerve threshold” remains unknown, Singer’s ultimate hypothesis- that peripheral nerves produce one or more factors which are essential for blastema formation and growth- laid the groundwork for future studies of what is now known as
the “neurotrophic” model of nerve support (Fig.3.2A). This neurotrophic hypothesis has since its inception served as the basis for studies of nerve dependence in the regenerating salamander limb.

With one major mystery solved, focus shifted to the elucidation of other aspects of nerve dependence. A series of studies conducted by a number of researchers in the 1940’s and 50’s focused primarily on limb regression, a phenomenon first observed by Todd more than a century prior. If the salamander limb is denervated at the time of amputation, it typically undergoes extensive histolysis and tissue regression (Fig.3.2D, E). In certain cases, most often in larval animals, the denervated limb may even fall off completely (Butler & Schotté, 1949; Schotte & Butler, 1941). In 1953, Charles Thornton found a link between histolysis, denervation, and tissue injury when he conducted a study in which he crushed the radius and ulna of otherwise-intact limbs with a pair of watchmaker’s forceps. When the limb was fully innervated, this crush injury resulted in complete regeneration. However, if the limb was denervated at the time of injury, it underwent histolysis, inflammation, and tissue degradation so severe that the limb sometimes fell off at the site of injury. Only after complete regression to the shoulder and consequent reinnervation did the limbs finally regrow. Denervation of an intact limb, meanwhile, did not cause any appreciable change in its tissue architecture (Thornton, 1953). This study uncovered a link between denervation, injury, and inflammation, and few studies have attempted to follow up on it, indicating that this relationship may be an intriguing avenue for future research.

The discovery of nerve-independent salamander limb regeneration in the late 1950’s added an intriguing new dimension to the mystery of nerve dependence. In 1959, C.L. Yntema generated aneurogenic animals by removing the neural tube from embryos and found that the
limbs of these animals regenerated despite their lack of innervation (Yntema, 1959). This phenomenon was quickly confirmed in the following years, and in 1953 Steen & Thornton grafted nerveless limbs onto innervated host animals and examined their regenerative ability over time. Strikingly, the grafted aneurogenic limbs retained their ability to regenerate in the absence of nerves for several days after the surgery. However, the limbs lost their nerve-independence upon complete innervation from the host, and thereafter were incapable of overcoming denervation (Steen & Thornton, 1963). It has since been hypothesized that limbs become “addicted” to one or more regeneration-critical factors which are produced in the developing limb but later supplied only by the nerves upon innervation. One candidate factor is anterior gradient, which is expressed in the developing aneurogenic limb but decreased after grafting and innervation (Kumar et al., 2011).

A series of grafting and in vitro studies in the latter half of the 20th century implicated proliferating blastemal cells as a major target of the nerves. In 1977, Liversage & Globus showed that blastemas cultured in vitro proliferated only if they remained innervated by spinal cord implants (Liversage & Globus, 1977), while Goldhamer et al. found that implantation of dorsal root ganglia into the blastemas of denervated limbs rescued cell cycling, although it did not increase cell cycling in fully-innervated blastemas (Goldhamer et al., 1992). Further characterization of cell cycling in the blastema showed that denervation effectively halts mitosis in blastemal cells (Goldhamer & Tassava, 1987; Maden, 1978b), and thus the modern study of nerve dependence has largely focused on elucidating the links between peripheral nerves and blastemal growth (Fig.3.2F, G).

While the blastema is widely-accepted as a probable target of peripheral nerves, other tissues remain more controversial. Because the epidermis is extensively invaded by nerves after
amputation (Singer, 1949; Taban, 1949; Thornton, 1954), a number of researchers have suggested that the apical epidermal cap (AEC) formed after amputation is dependent on innervation. Indeed, studies have found that the expression of $Dlx-3$ and $Sp9$, both of which are expressed in the regenerating wound epidermis, is nerve-dependent (Mullen et al., 1996; Satoh et al., 2008). However, during his groundbreaking early neurotrophic studies, Singer ablated sensory nerves from the wound epidermis and showed that limbs can regenerate with motor nerves alone (Singer, 1946). Later on, Sidmer and Singer showed that removing the sensory supply and preventing innervation of the epidermis still resulted in regeneration (Sidman & Singer, 1960), a finding that was supported by a concurrent Thornton study which demonstrated that epidermal innervation is not essential for the formation of the AEC (Thornton, 1960b). Finally, Singer and Inoue found that denervation does not significantly alter the morphology of the AEC, and they also managed to generate animals which exhibited a heavily-innervated wound epidermis but could not regenerate (Singer & Inoue, 1964). While these results do not preclude the possibility of paracrine signaling from mesenchymal nerves to the epidermis, they do call into question whether the direct innervation of the epidermis is critical for regeneration.

A 1975 study also suggested that one target of the nerve may be the vasculature, as denervation was found to block angiogenesis in the regenerating newt limb (Smith & Wolpert, 1975). Therefore, although it is clear that peripheral nerves are critical for cell cycling in the blastema, the relation of nerves to tissues beyond the blastema remains unclear.

**Molecular mechanisms of nerve dependence**

Despite this long history of the study of nerve dependence, many of the mechanisms underlying this process remain poorly-understood, largely because many modern techniques
have not been optimized for use with salamanders. Nevertheless, more recent approaches have strongly supported the notion that nerves supply one or more growth factors which are crucial for blastemal proliferation. Implicated factors include substance P (Anand et al., 1987; Globus et al., 1991a), insulin (Globus, 1978; Vethamany-Globus & Liversage, 1973a, 1973c), transferrin (Kiffmeyer et al., 1991; Mescher et al., 1997), nerve growth factor (Grillo et al., 1977; Weis & Weis, 1970), and fibroblast growth factors with BMPs (Albert et al., 1987; Christensen et al., 2001; Makanae et al., 2013; Mescher & Gospodarowicz, 1979). Much of this work has been conducted on cultured blastemal cells, and together these findings suggest that there is a constellation of factors working in conjunction to communicate proliferative signals from the nerve to the regenerating blastema. For a more detailed overview of these studies and their findings, see Table 3.1.

In 2007, Kumar et al. rescued regeneration in denervated newt limbs via electroporation of newt anterior gradient (Kumar et al., 2007b). However, it is not known whether anterior gradient plays a similar role in the commonly studied axolotl because there are surprising differences in the regenerative capabilities of axolotls (members of the family Ambystomatidae) and newts (of the family Salamandridae), which may have diverged more than 145 million years ago (Zhang & Wake, 2009). Most notably, newts appear to be incapable of recovering from denervation and are permanently unable to regenerate after a single denervation surgery (Liversage & McLaughlin, 1983). This stands in contrast with the axolotl, which is capable of re-innervating the limb and regenerating even after repeated denervations (Schotte & Butler, 1941). Moreover, adult newts appear to demonstrate a divergent method of muscle regeneration as compared to axolotls. Larval newts, like both larval and artificially metamorphosed axolotls, regenerate muscle via the recruitment of stem cells (Sandoval-Guzman et al., 2014). However,
adult newts switch to muscle fiber dedifferentiation after reaching adulthood (Tanaka et al., 2016), whereas axolotls are neotenic and do not undergo metamorphosis unless forced to do so via treatment with thyroid hormone. To our knowledge, there have not been any studies of nerve dependence in artificially metamorphosed axolotls or in closely related tiger salamanders (Ambystoma tigrinum), raising the possibility that newts and axolotls demonstrate contrasting methods of limb regeneration because of the divergent evolution of molecular regenerative mechanisms, differences in developmental maturation programs, or a combination of both factors.

The nerve-secreted mitogen hypothesis has received further support in the form of a technique now called the accessory limb model (ALM, Fig.3.2C). First described by Locatelli in 1929 (Locatelli, 1929) and first thoroughly characterized with modern techniques by Endo, Bryant and Gardiner in 2004 (Endo et al., 2004a), the ALM elegantly demonstrates the necessity of peripheral nerves for blastema formation. Inducing a wound in the epidermis and then deviating a peripheral nerve beneath this wound results in the formation of a proliferating bump. Satoh et al.’s thorough characterization of the bump formed by this surgery concluded that it expresses blastema-specific markers such as prx-1 and msx-2, thus indicating that it is analogous to the blastema formed after limb amputation (Satoh et al., 2007). In keeping with prior research, which has shown that denervation of the limb during the late blastema stage does not impair limb patterning and instead results in the formation of a miniaturized limb (Powell, 1969; Schotté & Butler, 1944; Singer & Craven, 1948), the presence of a nerve is sufficient to promote proliferation but does not contribute to limb patterning or differentiation in this model. Instead, a fully-patterned limb forms only if posterior epidermal tissue from the contralateral limb is grafted onto the anterior wound site. Nevertheless, the formation of this “accessory blastema”
provides a compelling model for precisely examining the targets and molecular mechanisms of innervation during the process of blastema formation. A 2013 study by Makanae et al. showed that Gdf5 and FGFs are together capable of inducing blastema growth and limb formation in this model even in the absence of a deviated nerve (Makanae et al., 2013). Even more recently, Satoh et al. have shown that FGF8 and Bmp7 are expressed at the ends of peripheral nerves, and knockdown of these factors inhibits blastema formation in regenerating limbs (Satoh et al., 2016). These findings reinforce the validity of the AL model as an analogue to the amputated limb while demonstrating the necessity of FGFs and BMPs for nerve dependent limb regeneration.

Although there is strong evidence supporting the mitogenic hypothesis of axolotl nerve dependence, there are some indications that denervation results in more than just the loss of a critical proliferative signal. In 1998, Irvin and Tassava showed that implantation of axotomized peripheral nerves into amputated limbs slowed blastema formation, suggesting that denervated nerves secrete inhibitory factors which may block proliferation (Irvin & Tassava, 1998). Tassava also showed that implantation of axotomized peripheral nerves into aneurogenic limbs inhibited regeneration, further indicating the possibility of inhibitory factors secreted by peripheral nerves after denervation (Tassava & Olsen-Winner, 2003). These findings are not unprecedented, as studies in various animal models have shown that peripheral nerve injury—whether from crush, axotomy, or disease—induces significant inflammation via Schwann cell activation (for review see (Cámara-Lemarroy et al., 2010; Dubový et al., 2013; Ydens et al., 2013)). While more study in this area is needed, it is possible that denervation of the amputated salamander limb inhibits amputation in two ways: 1) it results in a loss of nerve-derived mitogens that are essential for blastema formation and 2) it induces inflammatory signals from
injured nerves which then inhibit the formation of a regeneration-permissive cellular environment (Fig.3.2C).

**Neuregulin-1 as a nerve-derived blastemal mitogen**

Our recently published work (Farkas et al., 2016b) builds on this long history and illuminates some previously unexplored molecular mechanisms of regeneration by examining the role of Neuregulin-1 (NRG1), a nerve-derived mitogen, during axolotl limb regeneration. NRG1, which is known to have a role in both cardiac and peripheral nerve development (for review see (Falls, 2003b)), was first implicated in newt limb regeneration by Brockes and Kintner in 1986 (Brockes & Kintner, 1986), and again by Wang et al. in 2000 (Wang et al., 2000a). Via immunohistochemical and in situ analysis, we found that NRG1 and its active receptor ErbB2 are expressed in the regenerating axolotl blastema. While NRG1 protein was expressed in dorsal root ganglia and peripheral nerves as well as the mesenchyme and wound epithelium in the preblastema stage of regeneration, it was particularly highly expressed in the proliferating blastema and in fact colocalized with a majority of proliferating blastema cells, as assessed by BrdU incorporation. Denervation of the limb resulted in a significant decrease in NRG1 and ErbB2-positive cells, suggesting that NRG1 signaling is dependent on the presence of nerves.

In order to determine whether NRG1 is capable of bypassing the presence of nerves to induce regeneration, we implanted NRG1-soaked beads underneath the wound epithelium of denervated limbs at 6 days post amputation (DPA). It must be noted that denervation and bead implantation occurred on day 6, after the initial inflammatory processes had concluded. This mirrors the 1987 results from Tomlinson and Tassava, which showed that denervation followed by dorsal root ganglia implantation rescued regeneration at 10 and 14 DPA, but never at 1 DPA.
(Tomlinson & Tassava, 1987). It is therefore possible that NRG1 supplementation is insufficient to fully rescue denervation starting from day 0, as it is apparently crucial for blastema formation and growth but may not be involved in earliest steps of the regenerative process such as dedifferentiation and wound healing.

In addition to rescuing limb regeneration, we also inhibited regeneration in fully innervated limbs via the specific ErbB2 inhibitor Mubritinib. Treatment with 500nMol Mubritinib substantially reduced proliferation at the amputation site and blocked regeneration at both 0 and 16 DPA. Inhibition of EGFR, which can heterodimerize with ErbB2 and signal through EGF, reduced epidermal proliferation and produced a markedly different phenotype from ErbB2 inhibition. We therefore concluded that NRG1 signaling through ErbB2 is a crucial upstream nerve-derived proliferative signal during initial blastema formation as well as blastema proliferation. Further research will be needed in order to determine NRG1’s role amongst the vast array of candidate factors discovered throughout the past decades of research.

Our findings have broad implications that extend beyond the field of salamander research. While it has long been known that nerve dependency is a phenomenon which extends across phylogeny, it has recently become increasingly clear that NRG1 itself plays a crucial role in regeneration across species and tissues as well. Although our study was the first to examine the role of NRG1 in axolotl limb regeneration, recent studies have implicated it in the regeneration of both peripheral nerves (Fricker et al., 2011; Ronchi et al., 2013; Ronchi et al., 2015) and the mammalian heart, with the latter topic gaining considerable recent interest. Bersell et al. found in 2009 that injection of NRG1 induced cardiomyocyte proliferation and injury repair in adult mice (Bersell et al., 2009). A subsequent study by Gemberling et al. in 2015 found that overexpression of NRG1 in zebrafish cardiomyocytes promoted cardiac
regeneration and induced substantial cardiac proliferation and growth in uninjured animals (Gemberling et al., 2015), while another recent study found that administration of NRG1 rescued regeneration in denervated neonatal mouse hearts (Mahmoud et al., 2015). Research into the role of NRG1 for heart regeneration continues, and it is increasingly clear that NRG1 signaling is crucial for a number of regenerative processes in animals ranging from fish to mammals.

NRG1 has also been implicated for its neuroprotective and anti-inflammatory effects in the central and peripheral nervous systems. NRG1 treatment attenuated neuroinflammation after stroke induction in rats (Xu et al., 2004), and administration of NRG1 has also been shown to protect dopaminergic neurons in a mouse model of Parkinson’s disease (Carlsson et al., 2011). Furthermore, NRG1 signaling is altered in patients with Alzheimer’s disease (Stefansson et al., 2002), and a recent 2016 study suggested that it is crucial for protecting cortical neurons against oxidative stress and damage (Jiang et al., 2016). This neuroprotective role seemingly applies to the PNS as well, as overexpression of NRG1 is sufficient to alleviate peripheral nerve demyelination in a mouse model of Charcot-Marie-Tooth disease (Fledrich et al., 2014b). It remains unknown whether NRG1 plays a role in attenuating the inflammatory response after axolotl limb amputation, and in fact the potential inhibitory effects of limb denervation are still largely unexplored in salamanders. NRG1 thus may play a variety of crucial roles during the process, and it stands as a promising candidate for the study of neuroprotective therapy in mammals.

Our findings are thus both a continuation of a long history of study and a new avenue for further research in the axolotl and beyond. As a novel upstream candidate factor for neurotrophic regeneration, NRG1 represents a substantial step towards solving the centuries-old mystery of nerve-dependent axolotl regeneration, and it may also represent a promising target
candidate for the future study of appendage regeneration and neuroprotection in non-
regenerating animals.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Relevant findings</th>
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<tr>
<td>Substance P</td>
<td>Increase in substance P-like immunoreactivity in peripheral nerves after amputation (Anand et al., 1987). Immunohistochemically found in the blastema and reduced after denervation; has a mitogenic effect on cultured blastema cells which can be blocked by adding substance P antiserum to nerve co-cultures (Globus et al., 1991a).</td>
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<tr>
<td>Insulin</td>
<td>A blastema does not form if the limb is amputated following pancreatectomy (Vethamany-Globus &amp; Liversage, 1973a). Insulin increases DNA and protein synthesis of blastemal cells in vitro; long-term exposure to insulin decreases the amount of time spent in G1 (Vethamany-Globus &amp; Liversage, 1973c). For review see (Globus, 1978).</td>
</tr>
<tr>
<td>Transferrin</td>
<td>Immunohistochemically found in both Schwann cells and axons; expression increases in nerves during limb regeneration and is decreased upon denervation; appears to be secreted by the ends of nerves after axotomy</td>
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<td>Factor</td>
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<tr>
<td><strong>Nerve growth factor</strong></td>
<td>Injections increased the length of the regenerate and the speed of digit formation (Weis &amp; Weis, 1970). Treatment with NGF increases the labeling index of dorsal root ganglia, as does amputation of the limb, although the effects are not combinatory (Grillo et al., 1977).</td>
</tr>
<tr>
<td><strong>Newt anterior gradient</strong></td>
<td>Expressed in the blastema and lost upon denervation; stimulates blastemal cell proliferation in culture; electroporation into denervated newt limbs rescues regeneration (Kumar et al., 2007b). Stimulation of blastemal cells can be blocked with a mutation to the anterior gradient active site or the addition of antibody to the receptor Prod1 (Grassme et al., 2016).</td>
</tr>
<tr>
<td><strong>Fibroblast growth factors and BMPs</strong></td>
<td>Stimulates the proliferation of cultured blastemal cells in a dose-dependent manner (Albert et al., 1987). Upregulated during regeneration and downregulated after denervation (Christensen et al., 2001). Injection into denervated blastemas shows dose-dependent stimulation of blastemal cell mitotic index (Mescher &amp; Gospodarowicz, 1979). Supplementation with FGFs and Gdf5 induces nerve-independent accessory limb formation (Makanae et al., 2013). FGF8 and Bmp7 electroporated into dorsal root ganglia are expressed at the ends of peripheral nerves; knockdown of Fgf8 and Bmp7 blocks blastema formation (Satoh et al., 2016).</td>
</tr>
<tr>
<td><strong>Neuregulin-1</strong></td>
<td>Present in the newt nervous system and the regenerating blastema; lost upon denervation; increases proliferation in cultured blastemal cells (Brockes &amp; Kintner, 1986). Expressed in the dorsal root ganglia and peripheral nerves of newts; injections into denervated newt blastemas induced regenerative growth (Wang et al., 2000b). Found along with its active receptor ErbB2 in the regenerative blastema and lost upon denervation; supplementation rescues regeneration to digits in denervated limbs; inhibition of ErbB2 signaling blocks regeneration (Farkas et al., 2016b).</td>
</tr>
<tr>
<td><strong>Oncostatin M and PDGF-AA</strong></td>
<td>Expressed in the regenerating mouse digit tip; supplementation rescues regeneration after denervation (Johnston et al., 2016).</td>
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Table 3.1. A brief description of the evidence for various candidate factors which have been suggested as essential nerve-derived mitogens during limb regeneration
Figure 3.1. Timeline of landmark nerve dependency studies throughout history, along with a selection of the most recent findings in the field.
Figure 3.2. (A) Diagram of the neurotrophic hypothesis, which states that nerve-secreted mitogens are transported from the dorsal root ganglia through the brachial plexus (BP) to support the proliferating blastema (BL). (B) Illustration of the inhibition hypothesis, in which denervation at the brachial plexus induces the release of inhibitory factors from Schwann Cells at the wound site. (C) Diagram of the accessory limb model, in which a peripheral nerve is deviated to a wound site to induce the formation of an accessory blastema (AB). (D) A control limb at 6 days post amputation (DPA). (E) A denervated limb at 6 DPA demonstrating extreme histolysis and inflammation. (F) The hyperinnervated regenerating blastema is highly proliferative at 14 DPA, as demonstrated by BrdU incorporation and staining. (G) Denervation eliminates beta-tubulin III staining of axons and substantially reduces the proliferative index of limbs at 14 DPA.
Chapter 4: Neuregulin-1 as a nerve-derived blastemal mitogen

As established in the previous three chapters, the axolotl is an ideal organism for investigating the molecular basis of nerve dependent regeneration. Building on the foundation of nearly two centuries of research, the most popular and well-studied hypothesis of nerve dependence has postulated that peripheral nerves supply one or more mitogenic factors which are necessary for blastema growth and formation. However, until recently no single protein was found to be capable of rescuing regeneration to digit formation in denervated limbs. Starting with its inception in the spring of 2013, the initial goal of the Monaghan Laboratory was to identify a protein that fit the characteristics of an essential nerve-derived factor. This candidate factor would ideally demonstrate the following characteristics: 1) it would be localized in the blastema and lost upon denervation 2) it would be capable of rescuing regeneration in denervated limbs, and 3) inhibition of the factor would block regeneration in fully innervated limbs.

The following paper examines whether the protein Neuregulin-1 (NRG1) fulfills these three qualifications. It was published in the journal Development in 2016 (Farkas et al., 2016c), and it represents the culmination of the early efforts of the Monaghan lab as well as the first half of my nerve dependence research. For this work we focused on NRG1, a mitogen that is known to play important roles in peripheral nerve development and cell proliferation, because it had been previously found in the newt peripheral nervous system (Brockes & Kintner, 1986) and one study had suggested that it may be capable of rescuing newt limb regeneration (Wang et al., 2000a). By localizing NRG1 via in situ hybridization and immunohistochemistry, supplementing denervated limbs with the secreted ligand of NRG1, and examining the effects of the pharmacological inhibition of the active receptor of NRG1, we were able to determine that NRG1 fulfills the three criteria listed above and acts as an essential mitogenic signal during
axolotl limb regeneration. Our findings, further explored and elaborated on below, indicate that nerve dependence is indeed a function of the secretion of a mitogens from peripheral nerves, thus providing a basis for the future study of blastema proliferation and nerve dependent cellular proliferation.

**Neuregulin-1 signaling is essential for nerve-dependent axolotl limb regeneration**

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**Summary Statement**

Denervation of the amputated axolotl limb prevents regeneration. We have found that the neuronally-secreted protein Neuregulin-1 is necessary for axolotl limb regeneration and capable of inducing regeneration in denervated limbs.

**Abstract**

The Mexican axolotl (*Ambystoma mexicanum*) is capable of fully regenerating amputated limbs, but denervation of the limb inhibits the formation of the post-injury proliferative mass called the blastema. The molecular basis behind this phenomenon remains poorly-understood, but previous studies have suggested that nerves support regeneration via the secretion of essential growth-promoting factors. An essential nerve-derived factor must be found in the blastema, capable of rescuing regeneration in denervated limbs, and its inhibition must prevent regeneration. Here we show that the neuronally-secreted protein Neuregulin-1 (NRG1) fulfills all these criteria in the axolotl. Immunohistochemistry and in situ hybridization of NRG1 and its active receptor ErbB2
found that they were expressed in regenerating blastemas but lost upon denervation. NRG1 was localized to the wound epithelium prior to blastema formation and was later strongly expressed in proliferating blastemal cells. Supplementation via implantation of NRG1-soaked beads rescued regeneration to digits in denervated limbs, while pharmacological inhibition of NRG1 signaling reduced cell proliferation, blocked blastema formation and induced aberrant collagen deposition in fully-innervated limbs. Taken together, our results show that nerve-dependent NRG1/ErbB2 signaling promotes blastemal proliferation in the regenerating limb and may play an essential role in blastema formation, thus providing insight into the longstanding question of why nerves are required for axolotl limb regeneration.

**Introduction**

Regeneration of the axolotl (*Ambystoma mexicanum*) limb is inhibited by denervation of the limb, but the molecular mechanisms underlying this nerve dependence remain largely unknown. Denervation of the amputated axolotl limb does not inhibit wound healing but blocks the formation of the post-injury proliferative mass called the blastema (Todd, 1823). Nerve dependence is a phenomenon observed during wound healing and regeneration across a wide range of phylogeny (Kumar & Brockes, 2012) and may be due to the secretion of essential growth-promoting factors from peripheral nerves at the wound site (Singer, 1952a; Stocum, 2011a). Though evidence has been gathered in support of numerous candidate factors including transferrin (Kiffmeyer et al., 1991; Mescher et al., 1997), fibroblast growth factors (Satoh et al., 2011) and anterior gradient protein (Kumar et al., 2007b), no neuronal factor thus far has proven capable of rescuing regeneration in the denervated axolotl limb. Here we examined Neuregulin-1 (NRG1), a neuronally-secreted mitogen that promotes proliferation through ErbB2 signaling (Falls, 2003b) and has been found in the newt peripheral nervous system (Brockes & Kintner,
1986) and implicated in newt limb regeneration (Wang et al., 2000a). We have shown that NRG1 is found in the axolotl peripheral nervous system and blastema, is capable of rescuing regeneration to the point of digit formation in denervated limbs, and that its inhibition inhibits blastema formation, suggesting that it is a vital upstream proliferative signal during the regenerative process.

**NRG1 and ErbB2 are expressed in regenerating limbs**

Blastema-specific expression of NRG1 isoforms and receptors (Fig.4.1A-1E) was observed via in situ hybridization (ISH) in blastemas collected at 16 days post amputation (DPA). Strong expression of the active EGF-like domain, which is found in all isoforms of NRG1, was observed in the mesenchyme as well as the basal wound epithelium of the blastema. Expression of the Ig-like domain of NRG1, which is common to types I and II NRG1, was strong in the distal mesenchyme and basal wound epithelium and was comparatively absent in cells located proximal to the site of amputation. The CRD domain of type III NRG1 was also observed in the blastema, though this expression was found in fewer cells compared to that of the Ig-like domain. Type I and type II NRG1 are capable of signaling in a paracrine manner while type III NRG1 is limited to juxtacrine signaling (Falls, 2003b), indicating that paracrine NRG1 isoforms in particular are strongly expressed in the blastema during limb regeneration. ISH of ErbB2 revealed that it was expressed in the mesenchymal cells of the distal blastema as well as the basal layer of the wound epithelium, though it was virtually absent from cells which were located proximal to the site of amputation. The ErbB2 co-receptor ErbB3 was similarly expressed in the distal blastema. Taken together, these ISH results suggest that expression of NRG1 and its receptors is blastema-specific during axolotl limb regeneration. RT-PCR of blastemal (21 DPA) and uninjured tissues found that all isoforms of NRG1 examined were
present in the regenerating and uninjured limbs (Fig.4.1F). NRG1 types I&II were upregulated in injured vs. uninjured limbs while type III NRG1 was more highly expressed in uninjured limbs. The presence of NRG1 in intact limbs is consistent with its known roles in Schwann cell and neuromuscular junction maintenance (Falls, 2003a; Sandrock et al., 1997).

Immunohistochemical staining of 16 DPA blastemas further confirmed the nerve-dependent presence of NRG1 and its active receptor in the regenerating limb. At 16 DPA, NRG1-positive cells were found both in the wound epithelium and in 56.18% of mesenchymal blastemal cells (Fig.4.1G, H). By contrast, the percentage of mesenchymal NRG1-positive cells was significantly reduced to 29.87% in denervated limbs (Fig.4.1G, I). These findings indicate that NRG1 protein is found in the blastema and reduced upon denervation, suggesting that nerves support a positive feedback loop which sustains NRG1/ErbB2 expression. NRG1 antibody specificity was tested via Western blot analysis, which showed a band at the expected size of 47kDa and demonstrated stronger band intensity in blastemal tissue as compared to denervated tissue at 16 DPA (Fig.4.1T). Immunohistochemical staining for the receptor ErbB2 was consistent with these findings, as ErbB2 was strongly expressed in both the mesenchyme and wound epithelium of blastemas at 16 DPA (Fig.4.1J) but reduced upon denervation (Fig.4.1G, K). Overall, these results show that RNA and protein of both NRG1 and ErbB2 are highly expressed in the blastema during axolotl limb regeneration.

Dorsal root ganglia, which are capable of rescuing regeneration if grafted into a denervated limb (Goldhamer et al., 1992; Kamrin & Singer, 1959; Tomlinson & Tassava, 1987), showed extensive NRG1 and ErbB2 staining (Fig.4.1M, O). NRG1 (Fig.4.1L) and ErbB2 (Fig.4.1N) were further observed in cross-sectioned peripheral nerves. NRG1 staining in the basal wound epithelium was observed before blastema formation and as early as 6 DPA
(Fig.4.1P). Costaining with BrdU showed that at 6 DPA, NRG1 was also present in a subpopulation of proliferating mesenchymal cells located just underneath the wound epithelium. Though the mechanism behind blastema formation remains poorly-understood, previous studies have shown that signals from the basal wound epithelium may work in conjunction with nerves to induce the accumulation of dedifferentiated mesenchymal cells at the site of amputation (Goss, 1956b; Goss, 1956c; Loyd & Tassava, 1980; Tassava & Garling, 1979). The pre-blastemal presence of NRG1 in the wound epithelium as well as the proliferating mesenchyme thus indicates that it may play an important role in blastema induction.

NRG1/BrdU costaining was further observed in 16 DPA blastemas (Fig.4.1Q). BrdU-positive mesenchymal cells costained with NRG1 in 65.89% of cells in control limbs and 46.55% of cells in denervated limbs (Fig.4.1S), indicating a pro-proliferative function that is consistent with its known roles in cell proliferation and survival (Canoll et al., 1996; Flores et al., 2000; Garratt et al., 2000). Furthermore, cells which costained with NRG1 and BrdU were observed along peripheral nerves near the site of amputation, (Fig.4.1R) suggesting that Schwann cells may also be secreting NRG1. Taken together, these immunohistochemical results show that NRG1 and its active receptor are localized in peripheral nerves and the proliferating blastema and thus may promote nerve-dependent blastemal formation and proliferation.

**NRG1 supplementation rescues regeneration in denervated limbs**

To determine if NRG1 supplementation is sufficient to rescue regeneration in denervated limbs, NRG1β-1 peptide-soaked beads were implanted underneath the wound epithelium of limbs at 7 DPA (Fig.4.2A). Supplementation with NRG1 induced blastema formation in six of seven denervated limbs (Fig.4.2B-D), which regenerated significantly more tissue than PBS-treated denervated limbs but not innervated limbs across a span of two weeks (Fig.4.2E).
Blastema formation was not the result of nerve survival or regeneration, as demonstrated by the fact that immunohistochemical staining for nerves was deficient in denervated and NRG1-treated limbs at 21 DPA (Fig.4.2F-H).

Because axolotl limbs cannot be reliably denervated for longer than approximately 20 days, in a separate experiment we denervated blastemas at 19 DPA and supplemented them with NRG1-soaked beads every four days in order to determine whether this treatment was sufficient to rescue limb regeneration all the way to digit formation. By 36 DPA, four of five NRG1-treated limbs had regenerated to the point of digit formation, while three of three controls and zero of three denervated limbs developed digits (Fig.4.2I-L, Fig.4.S1). NRG1-supplemented limbs regenerated significantly more tissue than PBS-treated limbs, although they did not regenerate to the same degree as the fully-innervated controls (Fig.4.2M), suggesting that greater NRG1 supplementation or the inclusion of additional factors may be necessary to achieve total rescue. Alcian blue staining demonstrated the presence of chondrogenesis in the new digits in control and NRG1-treated limbs (Fig.4.2N, O), while beta-tubulin III immunohistochemistry further confirmed the lack of nerves in both denervated conditions (Fig.4.2P-R).

NRG1 supplementation thus appears capable of bypassing the threshold of nerves required for blastema induction and limb regeneration, a finding with considerable implications towards explaining the longstanding question of nerve-dependent regeneration. While it has been found that application of Gdf5 and Fgfs can induce limb formation in an accessory limb model of axolotl regeneration (Satoh et al., 2011), this is the first example to our knowledge of a single protein rescuing regeneration in the denervated axolotl limb. These results suggest that NRG1 acts as an essential link between nerves and the blastema, as it promotes blastemal growth
and proliferation throughout the entire process of limb regeneration, from early blastemal growth to later digit formation.

**Inhibition of NRG1/ErbB2 signaling blocks regeneration**

NRG1 signaling was inhibited with the specific (Nagasawa et al., 2006; Ufkin et al., 2014) ErbB2 inhibitor Mubritinib. Submersion in 500nM Mubritinib did not impair wound healing but completely inhibited blastema formation in fully-innervated limbs, rendering them outwardly identical to denervated limbs (Fig.4.3A-C). Limbs treated with Mubritinib regenerated significantly less tissue than control but not denervated limbs (Fig.4.3M), lacked cellular accumulation at the wound site, and morphologically resembled denervated limbs (Fig.4.3G, H). BrdU cell counts of 12 DPA blastemas found that cellular proliferation was significantly decreased and in fact virtually abolished in drug-treated limbs (Fig.4.3I-J, N) despite the presence of healthy nerves, suggesting that the observed lack of proliferation was the direct result of ErbB2 inhibition rather than any inadvertent loss of innervation.

To further examine the similarities between denervated and Mubritinib-treated limbs, animals were bathed in 500nMol Mubritinib starting at 16 DPA, well after blastema formation. Previous studies have found that denervation after blastema formation substantially reduces cell cycling and proliferation (Goldhamer & Tassava, 1987; Maden, 1978a; Tassava et al., 1974) and results in the formation of a miniature limb (Powell, 1969; Schotté & Butler, 1944; Singer & Craven, 1948), suggesting that nerves are required for blastemal proliferation but not limb differentiation and morphogenesis. We found that Mubritinib inhibited blastemal growth but not limb patterning over a span of 12 days, inducing the formation of miniature limbs that were phenotypically similar to those formed as a result of late denervation (Fig.4.3D-F). Limbs treated with Mubritinib regenerated significantly less tissue than control but not denervated limbs.
(Fig 4.3O), underlining the similarity between denervated and ErbB2-inhibited limbs. Our inhibition experiments thus indicate that ErbB2 signaling is necessary for promoting blastema formation and maintaining blastemal proliferation during the early tissue growth and late tissue patterning phases of regeneration.

Long-term (23 DPA) exposure to 10µMol Mubritinib induced contraction of the wound epidermis similar to that observed in mice after injury (Dunn et al., 2013), contrary to the minimal wound contraction observed in control limbs (Fig 4.3K-L). Axolotl tissue regeneration is a typically scar-free process that occurs with minimal collagen deposition (Levesque et al., 2010; Seifert et al., 2012c), but extensive and aberrant collagen deposition was observed in the mesenchyme of Mubritinib-treated limbs. The phenotype observed here after long-term ErbB2 inhibition indicates a disruption of these scar-preventing programs and resembles the phenotype observed in amputated limbs after total macrophage ablation (Godwin et al., 2013).

As ErbB2 can also heterodimerize with EGFR, we pharmacologically inhibited EGFR in order to ensure that the effects of Mubritinib were due to NRG1 and not EGF signaling inhibition. Animals bathed in the specific (Goishi et al., 2003; Han et al., 1996) EGFR inhibitor AG1478 for six days post-amputation exhibited a markedly different phenotype from Mubritinib-treated animals, as EGFR inhibition resulted in improper wound healing and eventual tissue regression (Fig 4.4D-G). Strikingly, these animals also developed excessive numbers of iridophores after just ten days of treatment (Fig 4.4H). Furthermore, EGFR inhibition significantly reduced epidermal but not mesenchymal proliferation at 5-6 DPA, while ErbB2 inhibition significantly reduced mesenchymal but not epidermal proliferation (Fig 4.4A-C, I, J). These results suggest that inhibition via Mubritinib primarily blocks NRG1/ErbB2 signaling rather than EGF/ErbB2 signaling, which instead appears to play a critical role in wound healing.
and epidermal proliferation after amputation. Overall, our data show that NRG1/ErbB2 signaling is essential for limb regeneration and may play a vital role in preventing scar formation during this process as well.

**Conclusion**

We have shown that a single nerve-derived protein, Neuregulin-1, is capable of supporting blastemal growth and tissue regeneration up to the point of digit formation in the denervated axolotl limb. We propose that nerve-dependent NRG1/ErbB2 signaling is crucial for blastemal proliferation and may also be an essential component of blastema formation and scar-prevention programs. Although NRG1 is the first protein to our knowledge that is capable of rescuing regeneration to digits in the axolotl limb, these findings do not rule out the possibility of other factors playing a crucial role in this process. Newt anterior gradient protein has been shown to rescue regeneration in denervated newt limbs (Kumar et al., 2007b), and despite some prominent species differences between axolotls and newts- which demonstrate a different recovery response to denervation (Liversage & McLaughlin, 1983) as well as a phylogenetically unique method of regenerating muscular tissues (Sandoval-Guzman et al., 2014; Tanaka et al., 2016)- further exploration of the relationship between these two signaling pathways is necessary in order to fully characterize the underlying cause of nerve dependency in the axolotl limb.

Given the conserved role of NRG1/ErbB2 signaling in the peripheral nerves as well as the burgeoning evidence of its necessity in other animal models of cardiac (Bersell et al., 2009; D'Uva et al., 2015; Gemberling et al., 2015) and peripheral nerve (Fricker et al., 2011; Ronchi et al., 2013; Ronchi et al., 2015) regeneration, elucidating the function and mechanism of this signaling pathway in the axolotl may have far-reaching impacts on the field of regenerative medicine.
Materials and Methods

Surgical procedures

Leucistic axolotls (*Ambystoma mexicanum*) were bred and raised at Northeastern University according to (Farkas & Monaghan, 2015). Animals were anesthetized in 0.01% benzocaine and amputation was performed just proximal to the elbow joint. Recombinant human NRG1β-1 peptide (0.5mg/mL in PBS; PeproTech) was incubated overnight with Affi-gel 50-100 mesh agarose beads (Bio-Rad) according to (Niswander, 2008). An incision was made 1-2mm above the site of amputation, then two beads were probed with forceps through the incision until they rested underneath the wound epithelium. Animals were denervated one hour later. Two more beads were implanted, limbs were re-denervated at 14 DPA, and blastemas were imaged three times a week. Area regenerated was assessed blind to the experimental condition via the tracing of blastemas in ImageJ. For the digit rescue experiment, three beads were implanted into the base of blastemas at 19 DPA, and denervations were performed one hour post-implantation. Three more beads were added every four days, limbs were re-denervated at 27 DPA and collected at 36 DPA. All experiments were conducted with the approval of and in accordance with the Northeastern University Institutional Animal Care and Use Committee.

Drug treatment

Mubritinib (TSZ Scientific) stock solution (10mM in DMSO) was diluted in salamander housing solution to 500nM for juveniles (3.5-6cm SVL) and 10µM for adults (20-25cm SVL), which are more capable of tolerating the drug. Juvenile animals were bathed in Mubritinib starting at either 0 or 16 DPA, while adult animals were treated from 6-23 DPA before tissues were collected and prepared for immunohistochemistry. AG1478 (Tocris) stock solution (10mMol in DMSO) was diluted to 10µMol and animals were bathed in either 500nM Mubritinib, 10µM AG1478, or
10µM DMSO for six days prior to tissue collection. BrdU (20mg/ml, Sigma) was injected I.P. at 1mg BrdU/ 1g animal. Limbs were collected at 24 hours post-injection.

Immunohistochemistry and histology

Tissues were fixed in 10% neutral buffered formalin at 4ºC overnight, washed 2 x in PBS, incubated in 10% EDTA at 4ºC for 48hr, processed for paraffin embedding, and sectioned at 10µm. Sections were deparaffinized and hydrated, pressure-cooked in 10% citrate buffer for 20 minutes (Cuisinart Electric Pressure Cooker CPC-600), washed 5 minutes in PBS, blocked for 30 minutes in 1.5% normal goat serum, incubated at 4ºC overnight in primary antibody, washed, and incubated for 30 minutes at room temperature in secondary Alexafluor (Life Technologies) antibody, then mounted and coverslipped with Slowfade Diamond Antifade Mountant with DAPI (Life Technologies). Slides stained for ErbB2 were soaked for 30 minutes in 0.05% saponin (Sigma) then washed 3 x 10 minutes in PBS prior to the blocking step. Primary antibodies are listed in supplementary materials. Picrosirius (Polysciences, Inc.) and Masson’s trichrome (Thermo Scientific) stains were performed per the manufacturers’ protocol. Alcian blue staining was performed according to (Lee & Gardiner, 2012a).

RT-PCR analysis and in situ hybridization

Total RNA was extracted from uninjured and 21 DPA limbs (QIAGEN RNEasy kit), converted to cDNA template (Life Technologies Maxima H Minus First Strand cDNA Synthesis kit), and PCR amplified (2X PCR Master Mix; Thermo Scientific) with 10ng cDNA template and 0.5uM of isoform-specific primers. PCR products were cloned into pGEM-T (Promega), sequence verified (Genewiz), and vectors used to generate digoxigenin-labeled probes. Limbs were collected at 16 DPA and ISH performed on 35µm thick cryosections according to (Monaghan et al., 2012). See supplementary materials for primer sequences.
Western blot

NRG1 primary antibody was diluted to 1:10000; secondary was goat anti-rabbit HRP at 1:5000 (Jackson ImmunoResearch). Mouse anti-alpha tubulin (1:5000, Sigma) was used as the loading control and was detected with goat-anti-mouse HRP (1:5000, Jackson ImmunoResearch). All antibodies were incubated in 5% BSA in TBST.

Competing interests

The authors declare no competing or financial interests.

Author contributions

J.E.F. and J.R.M. designed the research. J.E.F., P.D.F., D.M.B., and J.L.W. performed experiments and provided materials. J.E.F. and J.R.M. analyzed the data. J.E.F. wrote the paper with contributions from J.R.M., D.M.B. and J.L.W.
**Figure 4.1.** NRG1 and ErbB2 are expressed in the PNS and the regenerating blastema. (A-E) ISH showing that NRG1 isoforms and receptors are expressed in the blastema at 14 DPA. Insets show sense controls. (F) rt-PCR analysis showing upregulation of type I and II NRG1 isoforms in regenerating vs. uninjured limbs. (G-K) NRG1 and ErbB2 are expressed in the mesenchyme of regenerating blastemas but lost upon denervation (n = 4 biological replicates each). Green and orange fluorescence is due to autofluorescent cellular debris. (L-O) NRG1 and ErbB2 are expressed in dorsal root ganglia and peripheral nerves. (P) NRG1 is expressed in the wound epithelium and mesenchyme of 6 DPA limbs along with proliferating BrdU-positive cells. (Q) Extensive NRG1 expression and colocalization with BrdU in a 16 DPA blastema. (R) NRG1 and BrdU colocalization along peripheral nerves in a regenerating limb at 16 DPA. (S) Denervation significantly decreases the percentage of BrdU/NRG1 colocalization in 16 DPA limbs (n = 4 biological replicates). (T) Western blot of NRG1 at 16 DPA showing a band at the expected size of 47kDa and greater band intensity in blastemal tissue relative to denervated tissue. Data is represented as mean +/- s.e.m., statistical analysis performed via Student’s t-test, ***p < 0.001, **p < 0.01, *p < 0.05. Scale bars at 100µm.
Figure 4.2. Supplementation with NRG1 rescues regeneration in denervated limbs. (A) Timeline of early NRG1 supplementation experiment. (B-D) Supplementation with NRG1 rescues regeneration in denervated limbs at 20 DPA. (E) From 6-20 DPA, NRG1-supplemented (n = 7) limbs regenerated significantly more tissue than denervated (p < 0.05, n = 7), but not control limbs (p > 0.05, n = 3). (F-H) NRG1-supplemented limbs regenerated in the absence of hyperinnervation. (I) Timeline of late NRG1 supplementation experiment. (J-L) Implantation of NRG1-soaked beads into denervated limbs rescues regeneration to the point of digit formation at 36 DPA. (M) From 19-36 DPA, NRG1-supplemented (n = 5) limbs regenerated significantly more tissue than denervated (p < 0.05, n = 3) and significantly less tissue than innervated (p < 0.01, n = 3) limbs. (N-O) Alcian blue staining showing digit formation in control and NRG1-treated limbs. (P-R) NRG1 induced growth and digit formation in fully-denervated limbs. Data is represented as mean +/- s.e.m, statistical analysis performed via one-way ANOVA with Tukey’s post-hoc. Scale bars at 100µm.
Figure 4.3. Inhibition of ErbB2 blocks regeneration, inhibits proliferation, and induces aberrant collagen deposition. (A-C) Inhibition of ErbB2 with 500nMol Mubritinib blocks blastema formation at 13 DPA. (D-F) Mubritinib application after 16 DPA blocks limb proliferation but not patterning and appears phenotypically similar to day 16 denervataion. (G-H) Picrosirius staining showing that 23 days of submersion in 10µMol Mubritinib results in contraction of the epidermis (e) and aberrant collagen deposition (c) in the mesenchyme, contrary to the minimal fibrotic deposition seen in control blastemas (b). Arrowheads indicate the contracted wound margin. (I-J) Masson’s trichrome staining of control and Mubritinib-treated limbs at 12 DPA show a lack of blastemal accumulation in the drug-treated limbs. (K-L, N) Treatment with Mubritinib does not reduce innervation but significantly decreases the proliferative index of amputated limbs (n = 5). (M) At 14 DPA Mubritinib-treated limbs (n = 5) had regenerated significantly less area than control (n = 8) but not denervated (n = 8) limbs. (O) Limbs that were either denervated (n = 8) or treated with Mubritinib (n = 8) at 16 DPA regenerated significantly less tissue than control limbs (p < 0.001, n = 7). Arrows indicate the planes of amputation. Data is represented as mean +/- s.e.m., statistical analysis performed via Student’s t-test, **p < 0.01. Scale bars at 100µm.
Figure 4.4. EGFR inhibition inhibits wound closure and is phenotypically distinct from ErbB2 inhibition. (A-C) Proliferating cells are localized to the mesenchyme in AG1478-treated limbs and the epidermis in Mubritinib-treated limbs at 6 DPA. Arrowheads indicate autofluorescent cellular debris and red blood cells, dotted lines indicate the boundary between the wound epidermis and mesenchyme. (D-G) AG1478 treated limb showing aberrant wound closure over time compared to control limb at 3DPA and 8 DPA. (H) Limb treated with AG1478 for ten days demonstrating aberrant development of iridophores (i). (I) Control limb treated with DMSO for ten days showing lack of iridophores. (J-K) Percentage of proliferating epidermal and mesenchymal cells in control, Mubritinib-treated, and AG1478-treated limbs at 6 DPA (n = 5 biological replicates each). Data is represented as mean +/- s.e.m., statistical analysis performed via one-way ANOVA with Tukey’s post-hoc **p < 0.01. Scale bars at 100μm.
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**Supplementary Table 4.1.** Primary antibodies utilized for NRG1 localization, proliferative index staining, and western blotting
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**Supplementary Table 4.2.** Primer sequences for NRG1 isoforms and receptors
Supplemental Figure 4.1. Supplementation with NRG1 rescues regeneration to digits in denervated limbs. (A-C) Three of three innervated limbs implanted with PBS-soaked beads demonstrated digit formation at 36 DPA. (D-H) Four of five denervated limbs implanted with PBS-soaked beads regenerated to the point of digit formation at 36 DPA. (I-K) Zero of three denervated limbs treated with PBS-soaked beads developed digits by 36 DPA.
Chapter 5: Nerves as both permissive and mitogenic controllers of limb regeneration

The work of previous chapters deals primarily with the neurotrophic hypothesis of nerve dependence, which is by far the most well-studied and widely supported hypothesis of this phenomenon. Our previous work has demonstrated strong evidence for the role of NRG1 as a nerve-derived mitogen that promotes blastemal proliferation. However, as mentioned in Chapter 3, a second and less prominent hypothesis postulates that there is a further component of nerve dependence. Namely, this “inhibition” hypothesis states that denervated nerves actively block regeneration by virtue of the secretion of inhibitory factors. This hypothesis is supported by an early study which suggested that implantation of axotomized nerve bundles slowed down regeneration (Irvin & Tassava, 1998), as well as histological observations of the denervated limb. Denervated limbs are highly inflammatory and in many cases undergo histolysis and tissue regression, suggesting that denervated peripheral nerves are not inert and instead issue signals which may disrupt the normally regeneration-permissive cellular environment. Our goal was thus to explore the possibility of this inhibitory hypothesis by grafting different tissues at the wound sites of both amputated limbs and limbs which had undergone accessory blastema surgery.

The following chapter is an early draft of an upcoming research manuscript that will be submitted to a research journal shortly. Through grafting experiments and electroporation, we have found that axotomized peripheral nerves block blastema formation in the accessory blastema model and either inhibit or significantly slow regeneration in amputated limbs. Moreover, our grafting studies indicated that this inhibition was unique to nerves and often resulted in aberrant limb patterning later in the process. This inhibition could be rescued in the
accessory blastema model via the overexpression of NRG1, suggesting that NRG1 may act as a “positive” factor that both promotes cell proliferation and attenuates the effects of “negative” inhibitory factors which induce aberrant leukocyte migration. These findings suggest a second, largely unexplored dimension to axolotl limb regeneration: namely that in addition to the loss of proliferative secreted factors from the nerves, denervation also blocks regeneration because it induces the release of inflammatory factors from the damaged peripheral nerves.

**Introduction**

Nerve dependent salamander limb regeneration is a phenomenon that has been studied for nearly two hundred years yet remains poorly understood. Regeneration of the axolotl (*Ambystoma mexicanum*) limb is characterized as nerve dependent because it does not take place if the brachial nerves innervating the limb are surgically severed (Todd, 1823). Instead, formation of the post-amputation proliferative mass called the blastema is completely inhibited, and regeneration does not move forward until the limb is re-invaded by intact peripheral nerves (Schotte & Butler, 1941). Although nerve dependence was first reported in the early nineteenth century, only recently have the molecular mechanisms of this process begun to come to light. The most well-studied and well-supported hypothesis of nerve dependence is the neurotrophic hypothesis, which states that peripheral nerves release critical mitogenic factors to support the formation and growth of the proliferating blastema. This hypothesis was first proposed in the 1950’s by the lab of Marcus Singer, and subsequent studies have implicated a range of factors as potential nerve-derived mitogens, including Neuregulin-1 (NRG1) (Farkas et al., 2016b; Wang et al., 2000a) and FGFs (Makanae et al., 2013; Satoh et al., 2016).

Although there is substantial evidence to support the neurotrophic hypothesis, there are some indications that peripheral nerves play multiple roles during limb regeneration. In 1998,
Irvin & Tassava showed that the implantation of axotomized peripheral nerve bundles slowed down the regeneration of amputated limbs which had been partially denervated (Irvin & Tassava, 1998). A followup study found that implantation also inhibited the regeneration of aneurogenic limbs (Tassava & Olsen-Winner, 2003), which are never innervated but remain capable of regenerating so long as they are not infiltrated by peripheral nerve fibers. Together these findings indicated that denervation may also generate a cellular environment that is not permissive for regeneration, an inhibitory hypothesis that is further supported by the release of inflammatory factors that is known to occur after nerve injury (for review see (Cámara-Lemarroy et al., 2010; Dubový et al., 2013; Ydens et al., 2013)). Because axolotl denervation involves the severance, not removal, of peripheral nerves, one possibility is that axotomized peripheral nerves survive denervation and subsequently release factors which inhibit blastema formation, either by directly combating the proliferative effects of intact nerves or by inducing aberrant inflammation and the eventual formation of a cellular environment that is not permissive towards regeneration.

Here we show that the contribution of peripheral nerves to regeneration is indeed twofold. In addition to providing mitogenic factors to the proliferation blastema, peripheral nerves also maintain a regeneration-permissive cellular environment which is disrupted by denervation. We have found that implantation of axotomized peripheral nerve bundles blocks accessory blastema formation and slows the regeneration of amputated limbs while inducing aberrant digit formation. Accessory blastema formation was rescued via NRG1 overexpression, and axotomized nerves were found to induce increased leukocyte migration in vitro. Taken together, these findings suggest that denervation of the axolotl limb inhibits regeneration in part via the release of inflammatory factors, a process which attracts leukocytes but can be attenuated by NRG1 signaling.
Results and Discussion

To first examine whether axotomized nerve bundles are capable of blocking regeneration, we utilized the accessory blastema model (ABM). Known also as the accessory limb model, the ABM is a model for blastema formation that does not involve amputation (Fig.5.1.A). Instead, an epidermal window is cut into the limb and a peripheral nerve is severed distal to the site and redirected to the window, ultimately resulting in the formation of a “bump” that proliferates for several weeks before receding (Endo et al., 2004b). Because the ABM involves only the formation of an epidermal wound and the redirection of a peripheral nerve, it offers strong support of the neurotrophic hypothesis of nerve dependency, and characterization of the bump formed by this surgery has shown that it is analogous to the blastema formed after limb amputation (Satoh et al., 2007). Consequently, it was considered an ideal starting place for the study of nerve inhibition because it is far less complex than limb amputation and allows for the precise examination of the interaction between damaged and intact nerves.

To this end, we grafted axotomized hindlimb nerve bundles into the wound site of limbs which had undergone accessory blastema (AB) surgery (Fig.5.1B,C). The cell bodies of nerves grafted from GFP donors into white hosts demonstrated strong fluorescence after 15 days post-injury (DPI, Fig.5.1.D), suggesting that axolotl peripheral nerve cells, likely including Schwann cells, are capable of surviving axotomy. This finding is notable because the fate of axolotl Schwann cells after denervation was previously unknown. Although axolotls readily accept grafts and do not demonstrate strong immune rejection of foreign tissues, subsequent implantations were performed using nerve bundles removed from the animals’ own hind limb in order to control for this possibility. We found that implantation of axotomized nerve bundles
blocked blastema formation in ten of twelve limbs (Fig.5.1.G, Table 5.1), suggesting that denervated nerves are capable of blocking regeneration either directly or indirectly by combatting the mitogenic signals released from intact nerves. To control for the possibility of mechanical inhibition induced by the grafting surgery itself, some nerve bundles were decellularized prior to implantation. Multiple freeze/thaw cycles in liquid nitrogen killed the grafted nerve cells, as demonstrated by a loss of GFP fluorescence soon after implantation, while maintaining the physical structure of the graft. Implantation of decellularized nerve bundles inhibited blastema formation in only two of nine limbs (Fig.5.1.D,E), suggesting that inhibition was due to cell secretion rather than mechanical obstruction. Masson’s trichrome staining of the implanted limbs demonstrated aberrant collagen deposition at the wound site and a lack of organized cellular accumulation at the wound site (Fig.5.1.J). By contrast, limbs which received decellularized implants formed blastemas that resembled control limbs, despite the presence of the nerve grafts (Fig.5.1.H,I). Moreover, limbs which were grafted with muscle tissue formed blastemas in eight of ten cases despite the fact that GFP muscle demonstrated fluorescence at least 16 days after injury, suggesting that this inhibitory phenomenon is not due to the introduction of live tissue but is instead tied to peripheral nerve-specific secretions.

These findings support the inhibition hypothesis and are consistent with the earlier study of Irvin & Tassava, which showed that muscle grafts, in contrast with nerve grafts, have no effect on regeneration (Irvin & Tassava, 1998). Furthermore, they align with previous studies of Schwann cell signaling after nerve injury, which have shown that Schwann cells dedifferentiate and release a cocktail of inflammatory signals in response to a loss of axonal contact. While it is possible that this inhibitory effect is derived from other cell types or a combination cell signals, such as those secreted by perineural fibroblasts, these findings in conjunction with previous
research suggest that denervation of the axolotl limb blocks regeneration because it induces the secretion of inflammatory factors from Schwann cells.

Having established that axotomized peripheral nerve bundles are capable of blocking blastema formation in the accessory blastema model, we then moved towards determining whether peripheral nerve implantations are capable of blocking or slowing down the regeneration of amputated limbs. To this end, we implanted either one or two hind limb nerve bundles at the wound site of freshly amputated forelimbs. As a control, some “sham” limbs were instead implanted with two decellularized nerve bundles. Implantation of two nerves slowed blastema formation and significantly reduced the growth of limbs after 17 and 39 days post amputation (DPA), as compared to control and sham limbs (Fig.5.2.A-J, P). Significance was not observed at 61 DPA, however, limb growth was highly variable, with some nerve-implanted limbs regenerating normally and most regenerating partially (Fig.5.2.K-L). Limbs which received one nerve implant did not significantly differ from control and sham limbs, but patterning irregularities were observed, suggesting that nerve independence is dose-dependent and double implants result in slower regeneration and greater digit patterning deficits than single implants. Both single- and double-implanted limbs regenerated significantly more tissue than denervated limbs at 39 and 61 DPA,

Limbs which received nerve implants demonstrated a variety of patterning defects, and double nerve implantation induced these malformations at a greater rate than single implants. When limbs were scored based on the degree of their digit patterning, single- and double-nerve implanted limbs were found to display substantially more digit irregularities than control and sham limbs (Fig.5.2.Q). Denervated limbs, by contrast, did not generally regenerate to the point of digit formation. While some double nerve-implanted limbs did not grow past blastema
formation even after 61 days, the majority of those that did demonstrated a menagerie of bizarre phenotypes and malformations ranging from limb bifurcation to digit truncation or deletion (Fig.S.5.1.A,B). Alcian blue staining confirmed that these limbs had skeletal malformations, in contrast with sham limbs which demonstrated normal patterning and skeletal growth (Fig.5.2.R).

Implantation of axotomized peripheral nerves at 17 days post amputation (DPA) significantly slowed regeneration at 39 and 61 DPA as compared to control and sham limbs (Fig.5.3.A-I). Nerve-implanted limb growth was statistically comparable to the growth of limbs which were denervated at 17 DPA. As with implants from 0 DPA, nerve implants at 17 DPA led to digit patterning errors (Fig.5.5.J). However, these irregularities were less extreme and generally involved digit deletion or duplication (Fig.S.5.1). By contrast, limbs which were denervated at 17 DPA but did not receive implants formed miniaturized limbs which were small but not generally malformed, indicating that a direct or indirect conflict between intact and damaged nerves produced the observed patterning anomalies. Alcian blue staining confirmed the correct patterning of sham limbs and revealed patterning deficits such as carpal fusion in one nerve-implanted limb which looked outwardly normal (Fig.5.3.K). To determine whether these or any of the 0 DPA digit abnormalities were permanent or an errant result due to abnormally non-regenerative animals, we re-amputated all limbs and followed their growth until 64 DPA. Only 2 of 68 limbs showed digit malformations upon re-amputation (data not shown), indicating that the nerve inhibition we observed was not due to any irregularities with the animals and did not persist upon re-amputation.

The implications of these findings are multifaceted. They support the earlier accessory blastema results and thus reinforce the hypothesis that injured nerves inhibit regeneration through the secretion of inhibitory factors which may directly or indirectly impede the formation
of a regeneration-permissive environment. Moreover, they demonstrate a previously unknown dimension of limb patterning in the context of regeneration. Although the molecular mechanisms of limb patterning remain largely unexplored in the context of limb regeneration, peripheral nerves are generally not believed to be major players in this process because late denervation results in the formation of a small but fully patterned miniature limb (Schotté & Butler, 1944). Here we have shown that a perturbation of peripheral nerve function at the time of amputation is sufficient to induce longterm growth and patterning deficits. Furthermore, implantation of axotomized nerves after blastema formation significantly slowed regeneration in a manner similar to denervation, but contrastingly resulted in the formation of digit abnormalities. This finding again suggests that denervation alone is not sufficient to cause patterning deficits, which may instead arise from competition between damaged and intact peripheral nerves.

This nerve implantation method therefore appears to represent an intermediary between denervation, which results in a complete loss of mitogenic signals, and normal regeneration which is unchallenged by aberrant levels of inhibitory or inflammatory factors. Intact host nerves which innervated the wound site of implanted limbs were still capable of inducing proliferation in a limited pool of cells, which may have supported growth to the point of digits but was not sufficient to fully regenerate a normally patterned limb. This presumption is further supported by the finding that later nerve implants induced patterning deficits which were generally less extreme than the deficits observed after early nerve implantation, likely because the regenerating limbs had a larger pool of proliferating cells with which to form digits than those which were challenged at the time of amputation. More studies are needed to clarify the
mechanism of this perturbation, which may be the result of redirected host peripheral nerves or aberrant angiogenesis in addition to the secretion of direct or indirect inhibitory factors.

To determine whether Neuregulin-1 (NRG1), a protein which we have previously found is a crucial nerve-derived blastemal mitogen (Farkas et al., 2016b), is capable of overruling the inhibitory function of axotomized nerves, we overexpressed NRG1 in limbs via electroporation before conducting accessory blastema surgery with nerve bundle implants (Fig.5.4.C). We chose to examine NRG1 both because of our previous work and because NRG1 is known to mitigate inflammatory Schwann cell signaling after nerve injury. Axonal overexpression of NRG1 was sufficient to rescue Schwann cell dedifferentiation in a mouse model of Charcot-Marie-Tooth disease (Fledrich et al., 2014a), and NRG1 treatment has been shown to attenuate the expression of known inflammatory factors such as interleukin-1beta and macrophage chemoattractant protein-1 (Xu et al., 2005). We observed that NRG1 was expressed in the accessory blastema but comparatively absent from cells near the wound site in nerve-implanted limbs, suggesting that NRG1 signaling is somehow disrupted by the factors release by peripheral nerves after axotomy (Fig.5.4.A,B). Consistent with previous studies, overexpression of NRG1, observed via co-electroporation with GFP, was in fact capable of rescuing blastema formation in this model (Fig.5.4.D,E). Eleven of sixteen limbs electroporated with NRG1 formed blastemas even in the presence of axotomized peripheral nerve grafts. By contrast, only four of thirteen limbs which received GFP alone developed blastemas (Table.5.2). While future studies are needed in order to identify the inhibitory signals secreted from denervated nerves, which may act through either direct or indirect means, these results suggest that NRG1 acts both as a mitogen and an attenuator of inhibitory signaling during early limb regeneration.
This apparent conflict between intact peripheral nerves, which are known to provide mitogenic signals to the blastema, and denervated peripheral nerves, which are evidently capable of blocking these proliferative signals, may help explain a long running curiosity of salamander nerve dependence. In the 1950’s and ‘60s, the lab of Marcus Singer conducted a series of experiments that ultimately resulted in the formulation of the neurotrophic hypothesis (Singer, 1952b, 1964). Singer concluded that there is a nerve fiber threshold for regeneration: if enough peripheral nerve fibers are present at the wound site threshold is met and regeneration takes place; if not, regeneration will not occur. The mechanism of this “all or nothing” feature of limb regeneration has remained unclear, but our findings here suggest that NRG1 plays a vital role in the process. Blastema formation may then require a molecular “switch” from early inflammatory signals released by peripheral nerves after amputation to mitogenic nerve signals such as NRG1. However, these results also suggest that the nerve threshold may be mutable and more complex than a fixed dichotomy, as we were able to induce a partial regenerative state in amputated limbs by challenging intact host nerves with injured nerve tissues.

We next decided to further explore the mechanisms of nerve inhibition by examining whether peripheral nerves induce the migration of leukocytes in response to axotomy. Denervated limbs are highly inflammatory and exhibit aberrant tissue histolysis and leukocyte infiltration (Fig.5.5.A,B). The role of the immune system during limb regeneration is highly complex and largely unexplored. Macrophages infiltrate the wound site shortly after amputation to clear debris and senescent cells (Yun et al., 2015), and ablation of macrophages completely inhibits regeneration (Godwin et al., 2013). However, they vacate the wound prior to blastema formation (Godwin et al., 2013), suggesting that they are unnecessary or actively inhibitory towards this process. To explore this possibility, we injected animals intraperitoneally with
thioglycollate and collected lavage fluid from the peritoneal cavity five days later. Wright’s-Giemsa stain showed that the cells collected were a cocktail of neutrophils and monocytes (Fig.5.5.C), as identified by (Seifert et al., 2012c). Denervated peripheral nerve bundles were collected and placed in the bottom well of a Boyden chamber, while collected leukocytes were placed in the top well to assess migration.

We found that after 24 hours of incubation, wells which contained peripheral nerves demonstrated twice as many migrated cells as those which did not (Fig.5.5.D). Importantly, cells were not detected by the assay in wells in which peripheral nerves but not leukocytes were added, indicating that the observed results were due to genuine cell migration and not the passive dissolution of peripheral nerve cells. These results are the first to our knowledge which show that axotomized axolotl peripheral nerves actively attract leukocytes, although at present we do not know which specific leukocytes are most strongly affected. These findings are consistent with a previous study which showed that cultured Schwann cells attract macrophages in vivo (Tofaris et al., 2002), and they may offer an explanation as to why NRG1 proved capable of rescuing regeneration in nerve-implanted limbs, as NRG1 is known to decrease neutrophil adhesion and infiltration (Wu et al., 2015). More studies are necessary to determine whether the inhibitory effects of axotomized nerve bundles arise solely due to aberrant leukocyte infiltration, or if nerves also secrete signals which actively inhibit the proliferation of blastemal cells.

**Conclusion**

Taken together, our findings suggest that contrary to the widely held view of axolotl nerve dependence, denervation of the axolotl limb impedes regeneration in two ways. Previous studies have showed that mitogenic factors which are normally secreted from peripheral nerves in support of blastema formation are lost after denervation (Fig.5.6.A). We have shown here that
there is a second role of peripheral nerves, which involves the maintenance of a regeneration-permissive environment. Upon axotomy, peripheral nerve cells, likely Schwann cells, release factors which attract leukocytes and render the wound environment impermissive to cellular proliferation, leading to either an inhibition of blastema formation or partial regeneration characterized by aberrant digit patterning (Fig. 5.6.B). Mitogenic factors that are released from intact nerves, such as NRG1, can overrule this inhibition in great enough quantities, providing insight into the molecular mechanisms of the nerve threshold requirement for limb regeneration. We can thus characterize limb denervation as both the loss of “positive” mitogenic factors and the gain of “negative” inflammatory factors. Future studies will elucidate the identity of these inhibitory factors and the precise mechanism by which inflammation impedes regeneration.

Methods

Accessory blastema surgery and tissue grafts
Leucistic animals approximately 12cm long were bred and raised at Northeastern University according to (Farkas & Monaghan, 2015). Accessory blastema surgery was performed as described by (Endo et al., 2004b). Briefly, animals were anesthetized in 0.01% Benzocaine and a window approximately 2x5mm was cut into the epidermis just proximal to the elbow joint. An incision was made distal to the wound and a brachial nerve was severed and redirected so that the cut end of the nerve rested within the epidermal window. To obtain axotomized nerve bundles, an incision was made in the hind limb and approximately 4mm of peripheral nerve tissue was removed. For muscle tissue, an incision was made into the hind limb and a piece of muscle approximately 4 x 2mm was removed. To decellularize nerve bundles, tissue were placed into a cryogenic vial, submerged in liquid nitrogen for 30 seconds, then placed in a 40°C water bath for 30 seconds. This cycle was repeated twice more and the tissue was then
implanted into the limb. Tissue grafts were placed into the epidermal window adjacent to the redirected nerve and secured by tucking one or both ends of the tissue underneath the epidermis. Animals were then kept out on moist Benzocaine-soaked kimwipes for approximately one hour post-surgery to facilitate wound healing. Limbs were imaged three times a week, then collected into 4% paraformaldehyde, cryoprotected in 30% sucrose, and embedded in OCT.

**Limb amputation and nerve grafting**

Limb amputation was performed just proximal to the elbow joint. Nerve grafts 6mm long were obtained as described above and either one or two bundle of tissue were inserted into the wound site. Denervation was achieved via surgical severance of two brachial nerves at the brachial plexus, while sham surgeries were conducting by implanting decellularized tissue grafts as described above. Late nerve grafts and sham surgeries were conducted at 17 DPA. An incision was made approximately 3mm distal to the end of the blastema, a tunnel was created leading into the blastema using forceps, and two nerve grafts approximately 6mm in length were inserted through the tunnel and into the blastema. Because the experiments were conducted concurrently, the same control animals were used for both. All limbs were imaged thrice weekly and blastema area was traced in ImageJ blind to the experimental condition.

**Electroporation**

Plasmids of pCAG-GFP and pCAG-EGF-like domain of NRG1+Secretion sequence were concentrated to 2000ng/uL and a 1:1:10 NRG1:GFP:fast green dye solution was injected into the limb at the approximate future site of surgery. Control limbs received only GFP:fast green solution. Limbs were then electroporated at 30 V with five pulses at a pulse length of 100ms. Green fluorescence was monitored and accessory blastema surgery was performed three days
later wherever GFP expression was deemed to be the strongest. Nerve grafting surgery then proceeded as described above.

**Immunohistochemical and histological staining**

Mounted limbs were sectioned Immunohistochemistry was performed as described previously (Farkas et al., 2016b). Masson’s trichrome was performed according to the manufacturer’s protocol.

**Alcian blue staining**

Alcian blue staining was performed as described by (Nguyen et al., 2017). Briefly, limbs were collected in 4% paraformaldehyde overnight, washed 3 x 5mins in PBS, placed in Alcian blue solution overnight, digested overnight in a 1% trypsin and saturated sodium borate solution, and cleared in successive overnight solutions of 3:1, 1:1, and 1:3 KOH:glycerol. Finally, limbs were imaged and placed in 100% glycerol for storage.

**Leukocyte collection and migration assay**

White blood cells were collected from animals as described by (Froese et al., 2005). Briefly, animals were injected intraperitoneally with 1mL of sterile thioglycollate medium. Five days later, animals were injected intraperitoneally with approximately 2mL of amphibian PBS (APBS). The animal’s abdomen was massaged to induce circulation of the injected APBS, which was then collected from the animal and spun down at 300G for 10 mins at 4°C. The pellet was resuspended in cold APBS, then cell concentration was assessed with a hemocytometer. Cells were spun down again and resuspended in complete amphibian L-15 medium (60% L-15 in diH20, 50µM beta mercaptoethanol, 100U antibiotic/antimycotic, 0.25% BSA ) for a final concentration of 1.6 x 10⁶ cells/mL. One limb was denervated, and 24 hours later the animal was
euthanized via submersion in 0.05% Benzocine followed by decapitation. Peripheral nerve bundles approximately 2mm long were immediately collected from the hind limb and placed into the bottom wells of a Boyden chamber (Cell Biolabs, 5µm pore size). Cell migration was then assessed per the manufacturer’s protocol, in triplicate for each condition with an incubation time of 24 hours. Migration was then measured with a spectrophotometer. Cell smears were stained with Wright Giemsa solution per the manufacturer’s protocol.
Figure 5.1 Implantation of axotomized nerve bundles blocks blastema formation after accessory blastema surgery. (A-B) Schematic showing the accessory blastema and nerve grafting protocol. (C) GFP nerve implant demonstrating fluorescence one day after AB surgery. (D) GFP fluorescence persists and is observed in cell bodies 15 DPI. (E-G) Blastema formation is blocked by peripheral nerve implantation unless the nerve is decellularized beforehand. (H-J) Masson’s trichrome staining showing that nerve implantation induces aberrant collagen deposition (blue) and a lack of organized cellular accumulation at 15 DPA. (K-M) GFP muscle implants retain fluorescence at 15 DPI but do not block blastema formation in the majority of cases. Significance was determined via chi-squared analysis.
<table>
<thead>
<tr>
<th>Condition</th>
<th>Blastemas formed/total surgeries</th>
</tr>
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<tbody>
<tr>
<td>Control AB</td>
<td>16/21</td>
</tr>
<tr>
<td>AB + decellularized nerve implant</td>
<td>7/9</td>
</tr>
<tr>
<td>AB + nerve implant</td>
<td>2/12</td>
</tr>
<tr>
<td>AB + muscle implant</td>
<td>8/10</td>
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Table 5.1 Implantation with axotomized nerve bundles, but not with muscle tissues or decellularized nerves, blocked blastema formation in the accessory blastema model.
Area regenerated (mm$^2$)

Day post-amputation

- Control
- D0 Denervated
- D0 Sham Surgery
- 1 nerve implant
- 2 nerve implants
Figure 5.2 Axotomized peripheral nerve implants at the time of amputation DPA inhibit limb regeneration. (A-O) Limbs implanted with two denervated nerve bundles directly after amputation (n=10) demonstrated significantly less tissue growth at 17 and 38 days as compared to control (n=9) and sham (n=8) limbs. Limbs which received a single nerve implant (n=8) were not significantly smaller than control or sham limbs but demonstrated digit abnormalities in some cases. (Q) Scoring of limb patterning at 61 DPA showed that nerve-implanted limbs displayed more digit aberrations than control, sham, or denervated limbs. Denervated limbs in most cases did not form digits. A score of 1 = no visible digits or significant growth beyond the blastema, 2 = digits were present but abnormal in size or patterning, 3 = patterning was outwardly normal. (R) Representative Alcian blue staining showing skeletal abnormalities in a double nerve-implanted limb. Limbs that underwent sham surgery showed digit patterning and skeletal development comparable to control limbs. Data is represented as mean +/- s.e.m., statistical analysis performed via Kruskal-Wallis test with Dunn’s post-hoc.
Area regenerated (mm$^2$)

Control
Late denervation
Late sham
Late nerve

Days post-amputation

5  15  25  35  45  55

0.0  5.0  10.0  15.0  20.0

39 DPA
A  B  C  D

E  F  G  H
61 DPA
Figure 5.3 Axotomized peripheral nerve implants at 17 DPA inhibit limb regeneration. (A-I) Limbs implanted with denervated nerve bundles at 17 DPA (n=10) demonstrated significantly less tissue growth at 39 and 61 DPA as compared to control (n=9) and late sham (17 DPA, n=7) limbs. Implanted limbs did not significantly differ in size from limbs which were denervated at 17 DPA (n=8). (J) Scoring of limb patterning at 61 DPA showed that nerve-implanted limbs displayed more digit aberrations than control, sham, or denervated limbs. A score of 1 = no visible digits or significant growth beyond the blastema, 2 = digits were present but abnormal in size or patterning, 3 = patterning was outwardly normal. (K) Alcian blue staining showing skeletal abnormalities in nerve-implanted limbs, including fused carpals and digit duplication. Data is represented as mean +/- s.e.m., statistical analysis performed via Kruskal-Wallis test with Dunn’s post-hoc.
Figure 5.4 NRG1 attenuates the inhibitory signals of damaged nerves. (A,B) NRG1 is expressed in the proliferative blastema formed after AB surgery, but is virtually absent in cells close to the wound site in limbs which receive axotomized nerve grafts. (C) AB surgery at 1 DPI demonstrating GFP at the wound site. (D,E) NRG1 overexpression rescues blastema formation in nerve-grafted limbs, and extensive GFP fluorescence is observed within the blastema.
<table>
<thead>
<tr>
<th>Condition</th>
<th>Blastemas formed/total surgeries</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP + NRG1</td>
<td>11/16</td>
</tr>
<tr>
<td>GFP</td>
<td>4/13</td>
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**Table 5.2** Overexpression of NRG1 via electroporation rescued blastema formation in the majority of nerve-implanted limbs which had undergone AB surgery. Significance was determined via chi-squared analysis.
Figure 5.5 Denervated axolotl peripheral nerves attract leukocytes. (A, B) Masson’s trichrome staining of limbs at 8 DPA show that denervation at the time of amputation induces aberrant inflammation and tissue histolysis. (C) Cells collected from thioglycollate injection are largely comprised of neutrophils and monocytes, as shown by Wright-Giemsa staining of cell smears. (D) Adding denervated peripheral nerve bundles to the bottom wells of a Boyden chamber significantly increased leukocyte migration (P<0.05). Data is represented as mean +/- s.e.m., statistical analysis performed via one-way ANOVA.
Figure 5.6 Proposed dual model of axolotl nerve dependency. (A) Denervation prevents the transport of essential mitogens such as NRG1 from the dorsal root ganglia to peripheral nerves at the wound site, thereby preventing blastema formation by depriving cells of proliferative factors. (B) Denervation also induces peripheral nerve damage resulting in the release of inflammatory factors from Schwann cells and the subsequent aberrant infiltration of leukocytes (L), resulting in a cellular environment which is not permissive for regeneration.
Supplemental Figure 5.1 Peripheral nerve implantation induces patterning defects in otherwise-normal limbs. Defects can be induced by implanted one (A) or two (B) peripheral nerve bundles at the time of amputation, or by implanting two bundles at 17 DPA (C).
Chapter 6: Conclusions and Future Avenues of Research

The work described in the previous chapters lays the groundwork for future studies in the field of regenerative biology, and efforts are underway to further expand upon these findings. As mentioned previously, a key question moving forward is the identity of the inhibitory factors which are released by damaged peripheral nerves after denervation. While we have not definitively identified any such factors, we have a number of clues to work with. A nerve-derived inhibitory factor is likely upregulated in and secreted from Schwann cells after axotomy. Given that Schwann cells dedifferentiate after loss of axonal contact, the factor may be upregulated in dedifferentiated cells or might even induce the process in Schwann cells. It may also induce inflammation via immune cell migration or activation. Furthermore, based on our previous findings, it is likely that NRG1 signaling is antagonistic towards at least one of these inhibitory factors. In summary, the perfect inhibitory candidate factor would be secreted by Schwann cells after injury, associated with Schwann cell dedifferentiation and inflammation, and antagonized by NRG1 signaling.

One factor which may fulfill all of these characteristics is amphiregulin (areg). A ligand of the EGFR pathway, areg has long been viewed as a perplexing question in molecular biology. It exerts generally proliferative effects on cells in culture, but a handful of studies have also suggested that it is capable of inhibiting cell growth (Akutsu et al., 2001; Sherbet, 2011). This seeming duality has posed a problem for researchers, but areg remains poorly studied in vivo and it is possible that the vastly more complex in vivo environment drives multiple functions in this single protein. We believe that this may tie into the workings of the immune system, as areg is in fact associated with several immune functions. Areg upregulates the expression of inflammatory cytokines including interleukin-1 beta (Streicher et al., 2007), interleukin-6, and interleukin-8.
It is also expressed in classically activated, pro-inflammatory M1 macrophages but not in alternatively activated M2 cells (Meng et al., 2015). Evidence linking areg to Schwann cell dedifferentiation is comparatively sparse, but it is massively upregulated in DRGs after nerve transection (Nilsson et al., 2005). Moreover, overexpression of EGFR-associated downstream signals upregulates areg and induces Schwann cell dedifferentiation in mice (Napoli et al., 2012) but can be attenuated via axonal overexpression of NRG1 (Fledrich et al., 2014b). Finally, areg expression spikes rapidly after axolotl limb amputation but drops to baseline levels before blastema formation, suggesting that it may play an essential role during wound healing and cell dedifferentiation but is not necessary for blastema formation and proliferation (Smith et al., 2005b). Future studies with thus endeavor to determine whether amphiregulin is released by Schwann cells after limb denervation and if it is capable of inhibiting or slowing down regeneration in fully innervated limbs.

In addition to the limb regeneration studies described above, we have found that our work extends outwards to cardiac regeneration as well. During the pharmacological inhibition studies which were described in Chapter 4, we found that animals could not survive more than ten days in the ErbB2 inhibitor Mubritinib. Closer examination of these animals revealed that they had grossly distended hearts which struggled to beat at a normal rate. Further research into the role of NRG1 in the heart divulged a substantial link between NRG1, which is essential for heart development (Gassmann et al., 1995; Lee et al., 1995; Meyer & Birchmeier, 1995), and cardiac regeneration. A groundbreaking yet controversial study published by Bersell et al. in 2009 suggested that overexpression of NRG1 was sufficient to drive cardiomyocyte proliferation in adult mice (Bersell et al., 2009), a startling finding given the general belief that cardiomyocytes are terminally differentiated and incapable of proliferation in adult mammals. Further research
in recent years has supported this finding in zebrafish and neonatal mice. The overexpression of NRG1 induces cardiomegaly in zebrafish (Gemberling et al., 2015), and supplementation with a mix of NRG1 and nerve growth factor is sufficient to rescue heart regeneration in denervated neonatal mouse hearts (Mahmoud et al., 2015). Furthermore, NRG1 also appears to exhibit cardioprotective effects against ischemia, as it is secreted from cardiac endothelial cells after injury and reduces apoptosis in cardiomyocytes (Hedhli et al., 2011). It has thus quickly launched to the forefront of regenerative research, and it stands as a promising candidate factor in studies of cardiac protection and wound healing.

Our preliminary findings, described in brief below, are consistent with these published studies because they suggest that NRG1 plays a role during both axolotl heart development and regeneration. Hearts were collected from animals treated with 500nMol Mubritinib for 16 days (as described in Chapter 4), sectioned, and stained using a Masson’s trichrome kit. Mubritinib-treated hearts demonstrated grossly diffuse cardiomyocytes which appeared significantly smaller and less dense as compared to control hearts. Furthermore, the pericardium of drug-treated hearts was greatly enlarged and appeared in some places to be sloughing off entirely, suggesting that NRG1 plays a major role during axolotl heart regeneration and thus inhibition of this signaling pathway leads to aberrant heart development (Fig. 6.1A, B). Immunohistochemical analysis of developing hearts showed that NRG1 is found in some pericardial cells where it curiously co-localizes with a neuron-specific marker (Fig. 6.1C). It is also found in proliferating cardiomyocyte niches, as demonstrated by BrdU injection followed by collection and IHC 14 days later (Fig. 6.1D), further supporting the hypothesis that NRG1 is essential for axolotl heart development.
When adult hearts were injured via ventricular resection and collected 28 days later, NRG1 was found to be localized to proliferating niches just beneath the pericardium, indicating that it may be secreted by perivascular cells to support proliferation after injury (Fig. 6.1E). Finally, injection of 10uM NRG1 intraperitoneally into larval axolotls resulted in a significant increase in cardiac proliferation after ten days, as demonstrated by EdU injection and subsequent whole-mount staining (Fig. 6.1F, G). Although these results remain highly preliminary, they nevertheless combine to indicate that NRG1 is essential for promoting proliferation during axolotl heart development and likely regeneration as well.

While the further investigation of the role of NRG1 during cardiac regeneration has been bequeathed to a new member of the lab, our preliminary research on this matter has demonstrated that it is a promising avenue of continuing research. Our past findings and future endeavors thus combine to tell a story that is multifaceted in its approach and significant in its implications. We have found that Neuregulin-1, of late the focus of intense study in various organisms ranging from fish to humans, plays a variety of key roles during salamander limb and potentially salamander heart regeneration, in harmony with previous and contemporary studies of its molecular functions in nervous and cardiac tissues. NRG1, in light of our findings, is crucial for promoting both the promotion of blastema proliferation after limb regeneration and the attenuation of post-injury inflammation. It may also contribute to axolotl heart development and cardiomyocyte proliferation after cardiac injury. These findings, and the findings that doubtless lie in wait within the future, will inform research efforts in the burgeoning field of regenerative medicine, and may one day provide the blueprint for successful regenerative therapies in humans.
Figure 6.1 NRG1 is essential for axolotl heart development and possibly regeneration. (A) Masson’s trichrome stain of a control heart from a larval axolotl, demonstrating larger and more densely packed cardiomyocyte as compared to Mubritinib-treated hearts (B). (C) NRG1 is localized to pericardial cells as well as proliferating cardiomyocytes (D) during axolotl heart development. (E) NRG1 is localized to proliferating cell niches beneath the pericardium after ventricular resection. The large gash in tissue at the center of the image is the wound site. (F-G) Injection of NRG1 into the intraperitoneal cavity of larval axolotls resulted in increased cardiac proliferation, as demonstrated by whole-mount EdU staining in green.
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