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An Investigative Laboratory Exercise Examining the Cell Signaling and Regulatory Properties of Neurons in the Regenerating Forelimbs of the Axolotl *Ambystoma mexicanum*

Kristina S. Mead
Biology Department, Denison University, Granville, OH, 43023

Many students understand the electrical properties of neurons and can adequately describe the creation and transmission of electrical impulses. However, students often have difficulty when it comes to understanding how neurons have an equally important role in cell signaling. This latter function is crucial in the establishment of proper cell fate during regeneration. I have devised a lab that includes in its goals: 1) an investigation into the cell signaling role of neurons, 2) experience with non-lethal invasive surgery, 3) an opportunity for student-collected data, and 4) the chance to work with charismatic animals. In addition, the exercise provides insight into development because development and regeneration follow similar pathways. The lab also introduces the topic of stem cells. Finally, the eventual regeneration of the denervated limb can provide an opportunity to discuss the mechanisms of nerve repair.

A critical event in urodele limb regeneration is the formation of a blastema. This event requires an intact nerve supply. Nerves secrete a substance called the neurotrophic growth factor(s) that appear to stimulate the reentry of blastema cells into the cell cycle, through a complex series of signaling events. In this laboratory exercise, students examine this effect by amputating both front limbs, but denervating only one. They then compare limb regeneration under and exempt from nerve control within the same animal. Students control for denervation using a behavioral assay, and monitor limb growth for six weeks. All sixteen of the surgeries were successful, and all showed the expected difference between the denervated and the control limb.

Key words: axolotl; blastema; regeneration; dedifferentiation; neurotrophic factor; glial growth factor

Introduction and Rationale

The axolotl (*Ambystoma mexicanum*) is a large aquatic, neotenic salamander native to Lake Xochimilco, Mexico. Adult axolotls can reach 30 cm or more in length from nose to tailtip, and they can weigh as much as 300 grams. Axolotls and newts are unique among vertebrates in their ability to achieve perfect, complete regeneration of their limbs and spinal cord even as adults (Brockes and Kumar, 2002). All the component regenerating structures including dermis, epidermis, nerves, blood vessels, muscle, bone, etc. are indistinguishable from those of ordinary limbs. Most other vertebrates that can replace missing structures do so imperfectly or only as juveniles (Brockes and Kumar, 2002). The axolotl's ability to regrow new limbs may be due in part to their cannibalistic life style. In nature, urodele limbs are 'amputated' by predators and the resulting wound initiates the regeneration cascade.

Background

Normal regeneration proceeds as follows: During the first day after amputation, a layer of epidermis migrates across the limb stump to cover the wound surface (Figure 1*B*; Repesh and Oberpriller, 1978). This step is critical for limb regeneration, since close contact between the epidermis and the underlying muscle is essential for regeneration to occur. Following wound healing, the cells dedifferentiate to create a local population of less specialized stem cells. These dedifferentiated cells accumulate under the epidermis to form the blastema, a mesenchymal growth zone (Figure 1*C*). The blastema then proliferates and then elongates to form a cone or bud (Figures 1*D* and 1*E*). It is thought that during the late bud stage, regeneration becomes nerve independent. The tissue then forms a flattened paddle shape (pallette stage; Figure 1*F*), and then starts the process of redifferentiation (Figures 1*G* and 1*H*). During this process, the undifferentiated blastema cells stop dividing and start to take on specialized cell fates. The structures that are reformed are exact replicas of the missing structures.

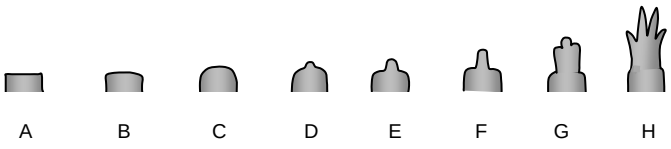

Figure 1. Simplified stages of regeneration in axolotl limb. *A*: amputation. *B*: wound healing. *C*: dedifferentiation (blastema). *D*: early bud. *E*: late bud. *F*: pallette. *G*: early redifferentiation. *H*: late redifferentiation.

The regenerating limb and the associated nervous tissue interact in a complex way, in both time and space. Experiments in which denervation occurs after amputation indicate that the ability of the blastema to reenter the cell cycle after dedifferentiation appears to be nerve dependent (Petroskey et al., 1980; Brockes 1984), but the latter steps of limb regeneration (late limb bud and beyond) appear to be nerve independent (Nye et al., 2003). That is, if nerves are severed early on in the regeneration process, the growth will regress. If the relevant nerve is severed late in

the process, regeneration will continue. Spatially, both the blastema and the nerves supply factors that are beneficial to the other tissue. Current thinking is that nerves supply an as yet unidentified factor termed the neurotrophic factor (NTF) that could be a glial growth factor, a fibroblast growth factor, a neural-derived transferring, or something completely different. Whatever the identity of the NTF, one of its functions is to stimulate cycling of the blastema cells (Wang et al., 2000). At the same time, the blastema cells secrete neurotrophic factors that stimulate reinervation of the blastema by sensory and motor fibers (Tonge and LeClere 2000). The exact identity of NTF is not yet known, but injecting a recombinant human glial growth factor intraperitoneally or into the blastema appears to stimulate the reentry of newt blastema cells into the cell cycle, suggesting that some newt GGF isoform is involved in this process in nature (Wang et al., 2000)

When a vertebrate motor neuron is severed, the distal portion of the axon degenerates (Nicholls et al., 2001). The Schwann cells that had formed the myelin sheath dedifferentiate and multiply, and other changes occur as well (Wallerian degeneration, Nicholls et al., 2001). Therefore, for a period of time after axotomy, there is no secretion of NTF. Within hours, new axonal sprouts begin growing threading their way through the column of Schwann cells. When they make contact with their target cells, Wallerian degeneration reverses and secretion of NTF can resume.

In this exercise, students amputate both axolotl forelimbs and sever the motor nerves controlling one of the two forelimbs during the same surgical procedure. By making careful measurements for several weeks after surgery, students can observe the difference in the way regeneration unfolds in the forelimb that is both denervated and amputated (no NTF) and the forelimb that is amputated but is still exposed to NTF. In this manner, students can come to appreciate the cell signaling role of the brachial nerves as well as their motor control function.

MATERIALS & METHODS

Animals

Mexican axolotls 8-15 cm in length *(Ambystoma mexicanum*) were obtained from the Indiana University Axolotl Colony — now moved to University of Kentucky and is now known as the UK Ambystoma Genetic Stock Center (AGSC). Animals were housed in individual gallon containers, fed soft salmon pellets (IUAC), and given new dechlorinated and conditioned (with Kordon's Amquel and Kordon's Novaqua) water containing 40% Holtfreter's salts three times per week as per the axolotl website (currently www.indiana.edu/~axolotl/). See Appendix for solutions and sources. This exercise was approved by Denison's IACUC (application #05-002).

Setup and surgery

Care must be taken to create a surgery station that is properly lit, ergonomically comfortable for the student, and relatively clean. See Appendix for details. The animals were anesthetized in 0.1% tricaine (methane sulfonate; MS-222) and placed under the microscope. A small hole was made in the epidermis slightly above and posterior to the shoulder and the tissue below was abraded to expose a landmark "Y-shaped" confluence of blood vessels (Figure 2). All three brachial nerves, which run superior-inferior just posterior of the blood vessel confluence, were severed. The layers of tissue were put back into place, but no stitches were necessary. The forelimbs were amputated using a new scalpel blade on each side. Any bone emerging from the stump was trimmed with scissors to ensure that a small flap of tissue surrounding the bone.

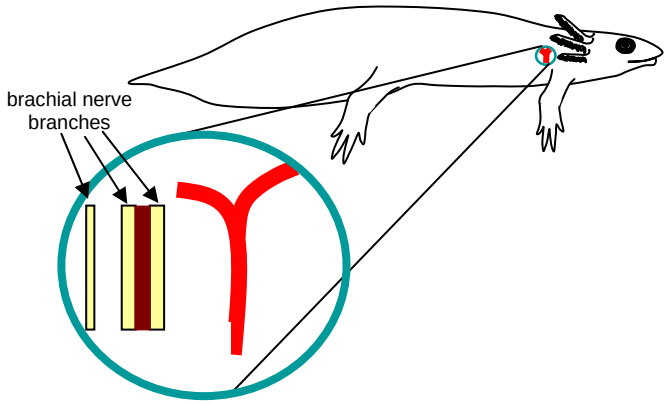

Figure 2. Anatomy of brachial nerves innervating forelimb. The three branches of the brachial nerve are posterior to a landmark Y-shaped branch in blood vessels

Behavioral assay

When the animal had recovered, the students checked for successful denervation by putting the animal in the "crawl tank" with 1 cm water. Animals unable to move the denervated limb were considered to have been successfully denervated.

Limb measurements

During the first year that we tried this lab, all nine groups of students monitored limb regeneration qualitatively only. The second year that this lab was done, all seven groups of students measured the extent of limb regrowth on both right (denervated) and left (control) sides using electronic calipers and a dissecting scope (when necessary). They also took pictures of both limbs using a digital camera. Measurements and pictures were taken once a week from week three to week six.

RESULTS

All nine groups who did the experiment the first year found slower regeneration in the denervated limb. Most control limbs were complete after six weeks, and the denervated limbs were complete three months after surgery, possibly earlier.

The following results pertain to the seven groups that followed regeneration quantitatively in year two. By 21 days post surgery, most control amputated forelimbs were in the mid- to late bud stages, while the denervated forelimbs were in the blastema to early bud stages (Figure 3*A*). One week later, the control forelimbs were in the

pallette to early differentiation stages, while the denervated limbs were in the early to mid-bud stages. By day 36, the control forelimbs were in early to late differentiation stages, and the denervated limbs were in mid- to late bud stages. By day 42, the untreated limbs were in late differentiation stages or were complete, and the denervated limbs were in the pallette to early differentiation stage (Figure 3*B*).

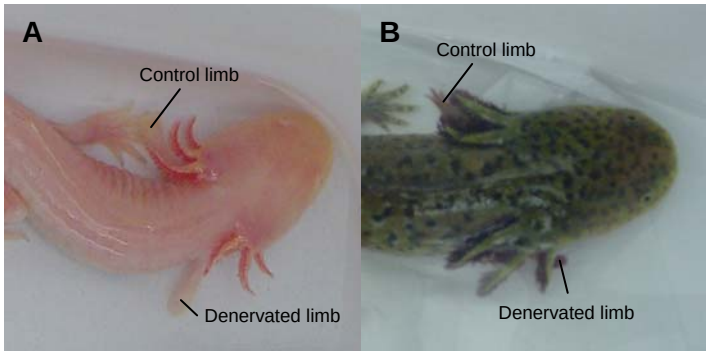

Figure 3. Axolotl 21 days (*A*) and 42 days (*B*) after surgery. In *A* the control limb is in the late bud stage and the denervated limb is in the blastema stage, and in *B* the control limb is in the mid- to late redifferentiation stage and the denervated limb is in the pallette stage.

In general, students found an increase in limb regrowth over time (Figure 4), a lag between the control and the denervated limb, and considerable variation among limbs within a treatment group at a given time point, despite statistically significant differences between treatment groups at every time point (Table 1).

In addition to demonstrating the role of the brachial nerve in supporting the regeneration of the forelimb (Figure 4 and Table 1), the lag time between the control and denervated forelimb regeneration in Figure 4 can provide a measure of the time required for the brachial nerve axon to find its way to the forelimb. Examination of Figure 4 and Table 1 suggests that the time required for the brachial nerve to heal itself (the lag time) must be about two weeks.

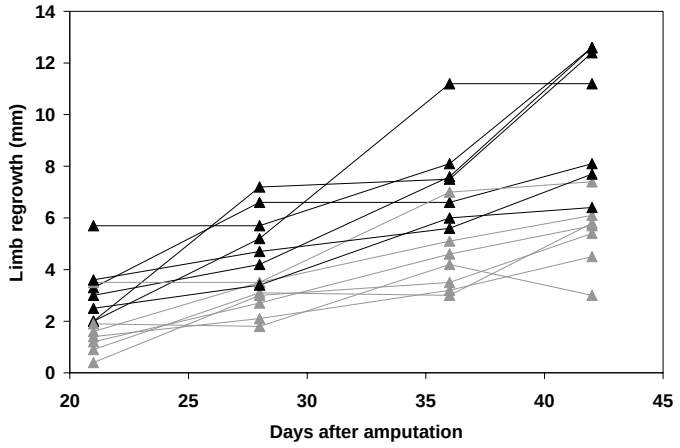

Figure 4. Comparison of regenerative limb growth between untreated (black) and denervated (gray) amputated forelimbs. Each datum is from a single animal. N=7 per treatment group.

Time since postop (days)	Mean length control limb (mm)	Stage	Mean length denervated limb (mm)	Stage	P-value from paired t-test
21	3.16 ± 1.28	D,E	1.56 ± 0.99	C,D	0.00018
28	5.41 ± 1.31	F,G	2.81 ± 0.66	D,E	0.0003
36	6.94 ± 2.52	G,H	4.37 ± 1.39	D,E	0.0337
42	10.14 ± 2.66	G,H	5.27 ± 1.34	F,G	0.0006

Table 1. Mean limb length ± standard deviations of the mean, regeneration stage (as described in the text and in Figure 1), and t-test comparing regeneration in untreated and denervated forelimbs at different time intervals post -surgery. N=7 per group.

This fits in with the observation that growth of nerve fibers is observed after about twelve days, with a roughly coincident increase in mitotic index (Petrosky et al., 1980). Given that the distance from the denervation to the stump is about two cm, the nerve must be regrowing on the order of 1 cm/week.

DISCUSSION

This lab has been used twice (with variations) in an Introduction to Neurophysiology class aimed at junior and senior biology majors with no previous neuroscience experience. Nine lab groups did the experiment the first year, and seven lab groups performed the manipulation the second year. All sixteen surgeries were successful (no deaths, complete loss of limb function on denervated side, regeneration on both sides albeit slower on denervated side). Although students were initially anxious about the surgery, they were very engaged and quickly overcame their squeamishness. Student comments were overwhelmingly positive. They included: “while we learned in class that animals that are capable of regenerating limbs do this better when the nerve is still intact, it was more interesting to witness it firsthand… this made the axolotl experiment one of the highlight labs in neurophysiology”, “At first I was hesitant to perform the surgery, for fear that something might go wrong, so that I would have left the axolotl permanently maimed. However, watching it heal itself over the subsequent weeks was almost miraculous, and took away any misgivings I had about the lab as a whole.” As well as reinforcing the concept of the role of the nerve in regulating regeneration, “watching the poor axolotl swim with one arm made me sad, but also solidified the concept of nervous stimulation of muscles.” Even though there was a lot of maintenance and data collection post-surgery, students didn’t seem to mind: “I enjoyed working directly with and looking after the live animals as well as being able to perform surgery on the animal in a way that wasn't brutal or permanently damaging.”

Materials that I have found to be helpful as references or as teaching resources are listed in the Appendix. Students of developmental biology might be stimulated to compare urodele limb regeneration, which relies on a mesenchymal growth zone, with urodele lens regeneration, which depends on the plasticity of epithelial cells of the iris (transdifferentiation; Okada, 1991; Mitashov, 1996).

While we performed the denervation and amputation on the same day, the procedures can be separated (Wang et al., 2000). Future experiments could examine the point at

which regeneration becomes nerve independent by denervating the forelimb at various time intervals after amputation. Experimenters could control for the effects of a pituitary derived hormone (such as prolactin) by hypophysectomizing axolotls (see Appendix). Other experiments could look at the effect of animal size, temperature, feeding regime, housing condition, or other factors on regeneration rate. Those with supplies of the appropriate antibodies could attempt to block the effects of NTF secretion by the intact brachial nerve, and those with access to large amounts of GGF could attempt to rescue regeneration in the denervated forelimb, similar to the experiments performed in Wang et al. (2000).

REFERENCES

Brockes JP (1984) Mitogenic growth factors and nerve dependence of limb regeneration. Science 225: 1280-1287.

Brockes JP, Kumar A (2002) Plasticity and reprogramming of differentiated cells in amphibian regeneration. Nat Rev Mol Cell Biol 3: 566-574.

Mitashov, VI (1996) Mechanisms of retina regeneration in urodeles. Int J Dev Biol 40: 833-844.

Nicholls JG, Martin AR, Wallace BG, Fuchs PA (2001) From neuron to brain, 4th edition. Sunderland, MA: Sinauer.

Nye HL, Cameron JA, Chernoff EA, Stocum DL (2003) Regeneration of the urodele limb: A review. Dev Dyn 226:280-294.

Okada, TS (1991) Transdifferentiation. New York, NY: Oxford.

Petrosky NS, Tassava RA, Olsen CL (1980) Cellular events in denervated limb stumps of Ambystoma larvae during re-innervation and subsequent regeneration. Experientia. 36:601-3.

Repesh LA, Oberpriller JC (1978) Scanning electron microscopy of epidermal cell migration in wound healing during limb regeneration in the adult newt, *Notophthalmus viridescens.* Am J Anat 151:539-555.

Tonge DA, Leclere PG (2000) Directed axonal growth towards axolotl limb blastemas *in vitro*. Neuroscience 100:201-211.

Wang L, Marchionni MA, Tassava RA (2000) Cloning and neuronal expression of a type III newt neuregulin and rescue of denervated, nerve-dependent newt limb blastemas by rhGGF2. J Neurobiol 43:150-158.

APPENDIX

Solutions

1) 100% Holtfreter's solution. Add 3.46 g NaCl, 0.05 g KCl, 0.1 g $CaCl_2$, and 0.2 g $NaHC0_3$ to 1 L dechlorinated tap water. (Can make and store as 10X solution). Use at 40% to house adults, and treat with Amquel and Novaqua before use.
2) 0.1% Tricaine (MS-222). To 1.8 L 40% Holtfreter's solution add 2 g tricaine. Adjust the pH to 7.2-7.4 with Na_2HPO_4 and add 40% Holtfreter's to make 2 L. Tricaine solution only lasts a few hours.

Reagent suppliers

Pre-mixed Holtfreter's salts (IUAC; Indiana University Axolotl Colony-now moved to the University of Kentucky; see Other Resource 2)
Food pellets (IUAC)
Kordon's Amquel (IUAC)
Kordon's Novaqua (IUAC)
Tricaine (Sigma-Aldrich A5040)

Surgical setup and procedure

Set up your station. You will need:
1 dissecting scope
1-2 lights
1 small sharp iris scissor from dissecting kit
2 sharp watchmaker forceps
1 Vannas scissors
1 squirt bottle with dechlorinated water to moisten the axolotl's skin
Microscope stage covered in paper towels topped by filter paper moistened in dechlorinated water

1) Clean off all of the dissecting equipment with moist kim-wipes (dechlorinated water)
2) Adjust seat height, lights, and focus (at the lowest magnification), and ensure that you have everything you need before putting the animal under.
3) Prepare a recovery chamber for your animal and fill it with fresh 40% Holtfreter's salts.
4) Anesthetize the animal in 0.1% tricaine (MS-222) in 40% Holtfreter's salts. When the animal can't right itself after being flipped over, it's out. This will take a few minutes, and can be preceded by wild swimming. (Note: a reviewer suggested another anesthetic: 0.007-0.01% para-aminobenzoate dissolved in 100% EtOH before being added to 50% Holtfreter's solution. Animals can be maintained submersed in this solution for hours.)
5) Place the anesthetized animal horizontally underneath the microscope on its left side with its head pointing right. The animal should stay under for about 20 minutes. Moisten the animal's skin if it starts to get tacky.
6) Move the gill out of the way.
7) Using watchmaker forceps, gently abrade away at the epidermis slightly above the right shoulder. Make a small hole/crescent.
8) Using the Vannas scissors and the forceps, expose a landmark "Y-shaped" confluence of blood vessels.
9) Slightly posterior to these vessels, you'll find the three brachial nerves running top to bottom (superior-inferior). You can tell you have nerve because it is glossy white, shiny, and often striated (a bit like the ridges on a CD, but not so iridescent). The two main branches of the brachial nerve surround a rather dark vessel. You may have to remove a couple of layers of connective tissue to see this. The third branch is much smaller than the other two. Cut all three branches of the brachial nerve.
10) Now amputate. Move the animal so that the limb you are going to cut is away from the gill and body. Cut with a new scalpel blade, right at the wrist. Now hold the animal up, and peer closely at the limb stump. There will probably be a little bit of bone, and you must snip it off using the small iris scissors. Your goal is to ensure that there is a little flap of tissue surrounding the bone. To do this, you will have to retract the sleeve of skin back, and cut the bone back while leaving the length of skin. This is very important for recovery and regeneration. Please make sure you do this thoroughly. Repeat on the other side and put the animal into its recovery chamber.
11) If desired, you can remove the possible influence of pituitary derived hormones by hypophysectomizing animals. While the axolotls are out, open the mouth and make a small incision in the roof of the mouth. Remove the cartilage above the pituitary. Apply slight suction using a Pasteur pipette with an angled, fire-polished tip to remove the pituitary.

Other Resources

1) Students read and discuss Wang et al. (2000) before lab.

2) The Indiana University Axolotl Center maintains a great website with detailed information about maintaining axolotls and including a newsletter and a helpful research tools page. The colony has moved to the University of Kentucky, but the website is still (temporarily) maintained by IU: www.indiana.edu /~axolotl/index.html.
3) Susan Bryant and David Gardiner's Leg Lab (darwin.bio.uci.edu/~mrjc/index.html) at UC Irvine has lots of great information on axolotl regeneration, including a time-lapse video at darwin.bio.uci.edu /~mrjc/Movie/movie.html.
4) For more detail on stages of normal axolotl limb development, see Nye et al. (2003).
5) The amphibian limb regeneration page at the Univ. of Guelph (www.uoguelph.ca/zoology/devobio/210labs/regen1.html) has great images of histological preparations of regenerating limbs.
6) As one of its Special Topic Booklets, Benjamin Cummings publishes *Stem Cells and Cloning*, by David A. Prentice (2003). The booklet is aimed at students and educators and includes a good list of web-based resources.
7) The Brockes and Kumar (2002) paper is an excellent review of different types of amphibian regeneration with many helpful references.

Received July 18, 2005; revised September 8, 2005; accepted September 15, 2005

I owe a great deal to Dr. Ken Klatt, who taught me the surgery, led me to some key papers, and read this manuscript. I thank the students in Introductory to Neurophysiology Spring 2004 and Spring 2005 for their careful axolotl care, data collection, and feedback on this exercise.

Address correspondence to: Kristina S. Mead; Biology Department, Denison University, Granville OH 43023. Email: meadk@denison.edu.