Sciatic Nerve Regeneration in *Hyla Versicolor*

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Introduction

Many types of neurons have the capacity to regenerate damaged axons. Studies have shown that two such neuron types are the afferent neurons of the frog optic nerve and the efferent neurons of the frog sciatic nerve. Regeneration of the frog optic nerve has been shown to be a relatively slow process with axonal outgrowths only extended 0.5-1.0mm in a 12 week period (Blanco and Orkand 1996). On the other hand, regeneration of the frog sciatic nerve is a much more rapid process with axonal outgrowth rates of 0.7mm/day to 1.1mm/day (Edbladh et al., 1989).

Here, in vivo regeneration of the sciatic nerve in adult male *Hyla versicolor* is studied. Percent regeneration as well as regeneration rates are determined for the motor axons of this nerve. These rates are then compared with those obtained in previous studies done on both efferent and afferent neurons in vitro and in vivo.

Materials and Methods

Animals

All of the *H. versicolor* used in this study were males collected from the UNDERC property in northern Wisconsin and the Upper Peninsula of Michigan between 25 June 2000 to 30 June 2000. They were kept in 10 gallon glass aquariums with no more than five animals per aquarium. Each aquarium contained approximately 2 cm of water and several tree branches. Animals were kept at room temperature (approximately 20°C) with exposure to natural photoperiods and with free access to insects caught daily on the property.

Surgical Procedure

All frogs (17) were anesthetized using between 2 and 3.5ml of benzocaine in 1000ml of cold tap water and ice. A small incision was made into their right thighs and their sciatic nerves

were exposed and crushed with forceps for approximately ten seconds. The incision was then stitched and the animal was placed on its back in a recovery tank until buccal movements were regained - at which time the animal was flipped onto its stomach and allowed to fully recover before being put back into a holding tank.

Approximately three weeks later all remaining animals (10) were sacrificed using 6ml of benzocaine in 1000ml of cold tap water and ice. Sections from both right and left sciatic nerves were removed from the knee to the high groin area. All nerves were immediately fixed in 4% paraformaldehyde in PBS and sent back to the University of Notre Dame in South Bend, Indiana.

Sectioning and Staining

All nerve samples were embedded in histo-prep and sectioned on a Reichert-Jung 2800 Frigocut N cryostat at 50μm. Sections were placed on subbed slides by alternating every other section per each slide (essentially creating two copies for each nerve). Slides were then placed in a desiccator for approximately 30 minutes and then stored at -20° C until they were stained.

Two staining procedures were used (one for each set of slides): Cresyl Violet (Humason 1972) and the Nauta and Gygax Method (Humason 1972). A few changes were made in the Nauta and Gygax Method. The ammonical silver nitrate solution was modified slightly due to an inability to obtain concentrated ammonia. Store bought extra strength ammonia was used in its place. Also, 10% paraformaldehyde in PBS was used in place of the 10% formalin for the reducing fluid. The Albrecht method was not used to mount the sections since sections were already on subbed slides. Slides were then cleared in heme-de and mounted with permount.

Analysis

Using a Nikon microscope at 100x magnification, the Cresyl Violet stained slides were used to determine the total number of axons for three sections on each nerve. These sections were each 700 μm apart. These same sections were then analyzed using a Nikon Eutectic Neuron Tracing System (NTS) to determine the cross-sectional area and diameter. The mean and S.E.M. were then determined for axon number, area, diameter and density of all nerves. An Anova test was also ran on each of these parameters. (Slides prepared from four of the animals were unable to provide data. Thus, the above analysis was run on the nerve slides prepared from the remaining six animals.)

Results

Table 1.

Parameters	**Proximal End**	**Middle**	**Distal End**
axon number	90.33±9.39	61.17±11.20	45±6.96
area (mm^2)	26.10±4.97	14.96±2.12	13.07±1.83
diameter (mm)	0.47±0.067	0.27±0.049	0.23±0.024
density (axons/mm)	3.67±0.55	4.43±0.78	4.11±0.95

The above table represents the mean and standard error of the mean for the total number of observed sections from all animal nerves. The number of axons decreases from the proximal end to the distal end. Area and diameter also decrease from proximal to distal. Density of the nerve (total number of axons/area of the nerve) does not follow this trend. Density actually remains constant within errors for the entire length of the nerve.

Multiple Anova tests were run on each of the above parameters (axon number, area, diameter and density). When an Anova was run on data from the proximal end, distal end and middle sections, the following p-values were observed: p-value for axon number equals 0.021; p-value for area equals 0.013; p-value for diameter equals 0.018; and p-value for density equals 0.826. Thus, significant differences exist in each parameter except density. Separate Anova tests were also run comparing data from the proximal end and distal end sections, the proximal end and middle sections, and the distal end and middle sections. Results for the proximal end and distal end Anova tests are as follows: p-value for axon number equals 0.0054; p-value for area equals 0.019; p-value for diameter equals 0.011; and p-value for density equals 0.731. These results show significant differences in all observed data except for that of the density. Results for the proximal end and middle sections are as follows: p-value for axon number equals 0.0986; p-value for area equals 0.0387; p-value for diameter equals 0.071; and p-value for density equals 0.491. These results show significant differences only in the area data. Results for the distal end and middle sections are as follows: p-value for axon number equals 0.289; p-value for area equals 0.55; p-value for diameter equals 0.364; and p-value for density equals 0.817. These results show that no significant differences are present between any of the parameters for the distal end sections and middle sections. Summarizing this data, it appears that the observed trend is significant differences are present in the two extremes but not in adjoining regions (with the exception of density which is consistent throughout the nerve).

Table 2.

animal number	number of axons disturbed	Number of axons regenerated	% regeneration	regeneration rate mm/day	number of days between surgery and sacrifice
11	16	9	56.25	0.02917	24
14	51	32	62.75	0.03333	21
16	75	7	9.33	0.03684	19
17	52	30	57.69	0.03684	19
42	20	16	80	0.03182	22
43	58	3	5.17	0.03333	21

The above table presents the percent regeneration and regeneration rates for each animal. The total number of axons disturbed was determined by the difference in axon number between the proximal end and distal end. The total number of axons regenerated was determined by the difference in axon number between the middle and the distal end. Percent regeneration refers to the total number of axons regenerated divided by the total number of axons disturbed in each nerve. The regeneration rate was calculated by simply dividing the distance between the proximal end section and middle section (700μm) by the number of days between surgery and sacrifice for each animal (19-24 days).

A survival rate of 58.82% was seen for the *H. versicolor* in this experiment. The actual cause of death for the seven animals lost was uncertain, but quite noteworthy nonetheless. The animals seemed to be suffering from some sort of seizure, which ultimately lead to drowning as the final cause of death. Two of these seizures were directly observed and such drowning was prevented. However, when 4 of the 7 lost frogs were found they were still in a tensed and contorted posture - lying drowned in the shallow water at the bottom of the holding tank.

Functionality of the right hind leg was observed after each surgery. Despite undergoing a 10 second nerve crush, not one of the 17 animals was unable to use its postoperative hind leg. All were able to climb and cling to the walls of the holding tanks in the normal preoperative positions. Also, there appeared to be no impedance in the animals' abilities to hunt food. In fact, the mobility of the hind leg became a small problem in a couple of the animals whose stitches became loose and who eventually acquired infections in the incision.

Discussion

Some assumptions were made in the course of this study, which should be addressed. It was assumed that the proximal end was the end of the nerve containing the greatest total number of axons and the distal end was the end containing the fewest total number of axons. When the nerves were removed from the animals they were not marked in any way – thus leaving the definite identification of proximal and distal ends impossible. It was originally thought that the area and diameter along the nerve would be constant from the proximal end to the distal end. However, Table 1 shows that the actual case consists of variable areas and diameters with constant density. This leads one to conclude that as the number of axons decreased the nerve shrank in size (similar to a balloon when air is released), thus keeping the density of the axons constant.

Results of the ANOVA tests ran show an important trend. Significant differences are present in the two extremes of the nerve (proximal end and distal end), but significant differences are not present in adjoining areas (with the exception of density which is consistent throughout the nerve). This suggests that change in the nerve from the proximal end to the distal end was consistent. No significant differences were seen moving from the proximal end to the middle or from the middle to the distal end.

Table 2 shows an extremely large range for percent regeneration (5.17% - 80%). However, both animals which had unusually low percent regeneration (animals 16 and 43) had unique circumstances. Animal 16 suffered from a severe infection in the incision and muscle surrounding the nerve. This may have had a negative effect on regeneration. Animal 43 died of natural causes and its nerve was removed within a maximum on 9 hours. It is possible that whatever caused the animals death also had a negative effect on regeneration in it's sciatic nerve.

Previous studies have shown that sciatic nerves crushed in vitro have an axonal outgrowth rate of 0.7mm/day to 1.1mm/day (Edbladh et al.,1989). These rates were determined using sciatic nerves removed from *Rana temporaria* and cultured prior to and after the nerve crush was made. Rates where determined by use of radioactive tracers. A similar study showed that in vivo responses to sciatic nerve crush approximately correspond to the in vitro values of ~8mm/day (Remgard et al. 1991). A direct comparison with these results and the results presented here is not possible since the exact same processes were not measured. The regeneration rates presented here represent the number of μm moved per day at a certain percent regeneration. They do not necessarily represent the actual distance moved per day by any one

axon, as do the other studies' results. Thus, when it is said that animal 11 has a regeneration rate of 0.02917mm/day at a percent regeneration of 56.25%, it does not necessarily mean that the axonal outgrowths were moving at a rate of 0.02917mm/day. Rather, it means that 56.25% of the disturbed axons regenerated and the average distance moved per day by these regenerating axons was 0.02917mm.

In vivo studies on regeneration have also been conducted using the frog optic nerve as a model. In one such study it was shown that the axonal outgrowth extended only 0.5-1.0mm in a 12 week period (Blanco and Orkand 1996). Also, at the end of three weeks (approximately the time of regeneration for the animals in this study) there appeared to be no outgrowth. Another study on optic nerve regeneration used recovery of prey acquisition as a measure of regeneration. Full recovery was seen between 11 and 16 weeks and signs of recovery were seen as early as 6 weeks (Singman and Scalia 1991). Theses results suggest that regeneration in the peripheral nervous system occurs much more rapidly in efferent neurons than it does in afferent neurons.

Future studies will need to be conducted using much larger sample sizes to confirm the data presented here. In such studies increasing the sample size is perhaps the most difficult task of the researcher as he or she must venture out into the field and catch animals for study. Especially when dealing with *H. versicolor*, various uncontrollable factors can determine the number of animals one is able to obtain for study. When working under time limits as in the UNDERC program, it may be wise to simply order the animals if conditions do not seem optimal for catching the animals in the field. Also, those conducting future studies will want to sacrifice the animals over a wider range of time. It would be advisable to label the proximal and distal ends of the nerve once it is removed from the animal so that no assumptions on which end is which need to be made. One should also attempt to remedy the problem of keeping the nerve straight up and down in the histo-prep prior to sectioning so that distance measurements and axon counts are more accurate. The Nauta and Gygax Method did not provide any significant data in this study. The changes made in the procedure may have something to due with this ineffectiveness. Another procedure, which may be beneficial for studying regeneration in peripheral nerves, is a modified Horseradish peroxidase technique (Tonge et al., 1992). This procedure has been shown to be especially useful in studying the early stages of regeneration in peripheral nerves.

Acknowledgements

I would like to thank Dr. Sunny Boyd for all of her help in both designing and conducting this study. I would like to thank Pat Maloney as well for his help throughout this study. Also, I would like to thank all of those involved with the University of Notre Dame Environmental Research Center (UNDERC) especially Dr. Jeff Runde and the Hank family.

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