The Role of Caenorhabditis Elegans Peroxidasin PXN-2 in Axon Regeneration

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Abstract

We investigated the effect of peroxidasin pxn-2 on axon regeneration in C. elegans. Peroxidasins comprise a gene family with homologues in C. elegans, Drosophila, and Homo sapiens. Peroxidasins are believed to contribute to the extracellular matrix, but the function(s) of peroxidasin proteins are currently unknown. We used RNA interference to display a mutant-like phenotype through pxn-2 knockdown in wild type and unc-70 worms. The unc-70 worms carry a ß-spectrin mutation rendering their axons unable to withstand mechanical strain, and hence they break in response to natural tensile forces incurred during movement. The axons of DD Gaba-ergic motor neurons of both wild type and unc-70 worm strains were tagged with green fluorescent protein (GFP), so the extent of regeneration in axonal commissures could be easily assessed. We found that the axon regeneration in the unc-70 worms that exhibited pxn-2 knockdown were significantly inhibited, with an average of 5.5 complete commissures compared with the average of 9.9 complete commissures found in the unc-70 worms with normal pxn-2 expression. With an average of 4.75 incomplete commissures and 4.23 missing commissures, the unc-70 worms affected by pxn-2 knockdown indicate that pxn-2 plays an important role in axon regeneration.

Introduction

One in fifty Americans suffers from some form of paralysis1. This amounts to over six million people with a permanent loss of muscle function and in some cases, a loss of sensory perception as well. The main cause of paralysis is spinal cord injuries, when a serious injury damages the spine in a way that severs the central nervous system axons. These broken axons are then unable to transmit information to and from the brain; therefore, any part of the body beyond the breakage cannot send or receive neural messages. There is currently no cure for paralysis, as damaged central axons in humans do not heal. However, some non-human species have the incredible ability to regenerate their central axons after they break2. Invertebrates, in particular, make excellent models for scientific study of axon regrowth since they can regenerate healthy and fully functional axons in a relatively short amount of time2. The challenge lies in identifying the genes that promote axon regeneration in invertebrates. If these genes can be isolated, and if they have human homologues, they would become potential therapeutic targets for the induction of axon regrowth in humans.

One promising gene family is the peroxidasin family, as recent research indicates that peroxidasin proteins play a role in axon regeneration3. Peroxidasins are relatively newly discovered genes with largely unknown functions, which make them a potential untapped resource for research. Furthermore, these genes are highly conserved across many species, including C. elegans, Drosophila, and, most importantly, Homo sapiens. Peroxidasins were first discovered in Drosophila in 1994 and characterized as peroxidase enzymes in the extracellular matrix (ECM)4. This finding was corroborated by further evidence of peroxidasins in the ECM of Drosophila myofibroblasts and fibrotic kidney cells5. Peroxidasins appear to reside in the ECM, but their function is unclear. Peroxidasins from Drosophila are known to be closely related to human peroxidase enzymes, so peroxidasins may catalyze hydrogen peroxide reactions such as radiiodination and oxidation in the same way that the glutathione, horseradish, and thyroid peroxidases do in humans6. This relation indicates that peroxidasin function may be conserved across species.

Potential roles for peroxidasins have been proposed, pointing to involvement in hydrogen peroxide reactions, consolidation and stabilization of the ECM, phagocytosis and apoptosis, and immune system defense4-6. However, these functions are still not definite, and certainly not exclusive. A recent study in C. elegans showed that peroxidasins promote embryonic development but inhibit axon regeneration1. The specific peroxidasin gene studied was pxn-2, which is homologous to the Drosophila peroxidasin and the human PXDN gene6. The same study showed that peroxidasins are located in the ECM and suggested that the substrate for peroxidasins may be type IV collagen in the basement membrane of C. elegans6.7. This exciting research highlights peroxidasins as a potential candidate gene for studies in search of novel therapies for spinal cord injuries.

In this experiment, peroxidasin pxn-2 was knocked down in C. elegans using RNAi by feeding to understand the role of pxn-2 in axon regeneration. A similar study indicated that pxn-2 should inhibit axon regrowth. In that study, laser axotomies were used to sever worms’ axons1. This current experiment utilizes RNAi sensitized unc-70 C. elegans with a ß-spectrin mutation that removes the axons’ ability to withstand mechanical strain. The worms that carry this ß-spectrin mutation naturally and more often than wild type worms. This genetic model for axon regeneration assays avoids the potentially confounding trauma of laser axotomy and should provide a better, more controlled environment to examine axon regeneration.

All experiments were conducted as post-embryonic developmental assays to avoid embryonic lethality and to focus on regeneration rather than normal axonal development. Wild type worms that possessed ß-spectrin served as the control group, and the DD motorneuron axons in these worms were tagged with GFP. L1 stage wild type and unc-70 worms were placed on RNAi plates with the pxn-2 RNAi feeding strain to assess the effects of pxn-2 knockdown in worms with and without the ß-spectrin mutation. In a separate experiment, unc-70 worms were fed either...
pxn-2 RNAi feeding strain bacteria or control bacteria to assess the effects of pxn-2 knockdown in worms with the β-spectrin mutation.

These experiments demonstrated that the knockdown of pxn-2 inhibited axon regeneration in unc-70 worms but had virtually no effect on wild type worms. The difference in the numbers of complete and incomplete commissures between the unc-70 worms that fed on control versus pxn-2 bacteria was statistically significant, suggesting that pxn-2 is a key component of axon regeneration. The function of peroxidasins in the ECM is still unclear, but we propose three hypotheses that address potential functional roles for peroxidasins in axon regeneration.

Materials and Methods

RNAi Experiments: HT115 feeding strains containing L4440 vectors encoding targets to pxn-1 and pxn-2 were attained for this experiment (a kind gift from Dr. Andrew Chisholm, University of California, San Diego). To re-streak the bacteria onto fresh LB/AMP/TET plates and LB/AMP plates, a protocol was used from the Dolan DNA Learning Center in the Cold Spring Harbor Laboratory at Cold Spring Harbor, NY. Interestingly, the pxn-2 feeding strain grew on both types of plates, the L4440 empty vector grew only in LB/AMP, and the pxn-1 failed to grow. The experiment was conducted without the pxn-1 since that feeding strain could not be salvaged. RNAi worm plates were seeded with the pxn-2 feeding strain, and glycerol stocks were prepared for later study. For RNAi experiments, C. elegans were cultured on LB agar plates that contained 10 mg/mL sterile ampicillin and 1M IPTG (Isopropyl-B-D-thiogalactopyranoside), according to a standard protocol also from the Dolan DNA Learning Center (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

C. elegans worms: In this experiment, wild type and unc-70 RNAi sensitized C. elegans with a β-spectrin mutation were used (a kind gift from Dr. Marc Hammarlund at Yale University). All worms used in this experiment had axons of DD Gaba-ergic motor neurons tagged with green fluorescent protein (GFP). Bleach protocol for unc-70 eggs: To study post-embryonic development, unc-70 C. elegans eggs were picked rather than L4-stage worms. However, these paralyzed worms laid very few eggs, so instead, the plates were bleached to collect eggs, following a protocol from the Journal of Visualized Experiments (JoVE). The worms and eggs were washed off of the RNAi plate with sterile water and a bleach/NaOH solution composed of 2.5 mL of bleach, 1 mL of 10N NaOH, and 6.5 mL of water was added. After the worms dissolved, the bleaching reaction was neutralized with 5 mL of M9 buffer. The remaining pellet of eggs was pipetted onto RNAi plates and allowed to hatch. However, when the RNAi plates were inspected later, no eggs were found. It seems that the unc-70 C. elegans were already so compromised by their β-spectrin mutation that the eggs were unable to withstand the bleach procedure. Microscopy: C. elegans were mounted onto agar padded slides and examined under an Axioplan Zeiss microscope. The agar padded slides contained 2% agarose and M9 buffer. Worms were examined using the GFP filter at 10x and 20x magnification and imaged using a Hamamatsu camera and Openlab software.

Results

In this experiment, we studied four groups of C. elegans: wild type worms feeding on control bacteria, wild type worms feeding on pxn-2 RNAi bacteria, unc-70 worms feeding on control bacteria, and unc-70 worms feeding on pxn-2 RNAi bacteria. To examine the role of pxn-2 in axon regeneration, we conducted two separate studies with these four worm groups. The first study compared wild type and unc-70 worms when both were exposed to pxn-2 RNAi bacteria, and the second study examined the effect of pxn-2 RNAi bacteria on unc-70 worms alone. Using the GFP filter on the microscope, complete and incomplete commissures were counted in L4 and adult stage C. elegans from each of these four worm groups. Figures 1 and 2 show examples of complete and incomplete DD commissures as seen through the GFP filter on a microscope. Figures 3, 4, and 5 are microscope images of the C. elegans in this experiment with GFP tagged commissures.

In the control group and in the wild type worms in pxn-2 bacteria, the median number of complete commissures was 15, and no incomplete commissures were observed. The wild type worms in control and pxn-2 bacteria had nearly identical numbers of commissures, with average numbers of 14.56 and 14.50 respectively, indicating that the pxn-2 bacteria had little to no effect on the worms. However, the unc-70 worms in control bacteria had an average of 9.90 complete commissures and 1.85 incomplete commissures, and the unc-70 worms in pxn-2 bacteria had an average of 5.55 complete commissures and 4.75 incomplete commissures; this suggests that the pxn-2 bacteria did have an impact on the unc-70 worms. Also, the unc-70 worms in control bacteria had an average of 11.75 complete and incomplete commissures, which is a lower average than the total commissures counted in the wild type worms. Assuming that C. elegans have similar total numbers of commissures, the unc-70 worms in control bacteria appear to be missing about 2.78 commissures, and the unc-70 worms in pxn-2 bacteria seem to be missing an average of 4.23 commissures. These missing commissures were most likely severed commissures that were unable to regenerate. The average numbers of counted commissures for the four worm groups are compared in Figure 6.

We then analyzed the data by making the box plots shown in Figures 7 and 8. The box plots display the range of the data: the middle 50% with gray boxes, called the Inner Quartile Range (IQR), and a median line in the middle of the IQR. The boxes on the dot plots represent outliers, which prevent a data set from being a “normal distribution”, which is a bell curve (an outlier is any data point greater than the 3rd quartile value plus 1.5 times the IQR or less than the 1st quartile value minus 1.5 times the IQR). The box plots found medians for the four worm groups to be 15 (wild type worms in control bacteria), 15 (wild type worms in pxn-2 bacteria), 10 (unc-70 worms in control bacteria), and 6 (unc-70 worms in pxn-2 bacteria), and the median coincided with the 3rd quartile value for wild type worms and unc-70 worms in pxn-2 bacteria. We also calculated standard deviations for the four worm groups to be 1.47, 1.00, 2.07, and 1.05, respectively, meaning the data for the unc-70 worms in control bacteria varied the most. In order to test the data for statistical significance, we must assume that the data follows a normal distribution (independent variables, symmetric data, and no outliers). Thus, it should be noted that...
this initial condition was not met, meaning the statistical tests may not be entirely accurate.

However, it is rare that a real data set follows a normal distribution, so we proceeded to run an analysis of variance (ANOVA test) on the four worm groups. The null hypothesis for the ANOVA test was that populations of the four worm groups should all behave the same way. The ANOVA test found that the probability that the different behavior of the four worm groups was due to chance was less than 0.0001, or 1 in 10,000. Therefore, we reject the null hypothesis, and the statistical evidence indicates that the different behavior of the four worm groups was not due to chance. Although the ANOVA test does not specify which or how many of the four worm groups behave differently, the test allows us to compare multiple groups of worms simultaneously and prevents us from rejecting the null hypothesis on false authority. Now, specific worm groups can be directly compared with the 2-sample T test, and the box plots help determine which worm groups are the best choices for comparison.

We performed the 2-sample T test on the number of incomplete commissures in the unc-70 worm groups, and this data set is visualized by the box plot in Figure 8. The plot found medians of 2 and 5 and standard deviations of 1.39 and 1.25 for the unc-70 worms in control bacteria and pxn-2 bacteria, respectively. The null hypothesis for the 2-sample T test was again that all of the unc-70 worms should behave the same way. The 2-sample T test found that the probability was also less than 0.0001, but this time, we can assert with statistical evidence that these two groups behaved differently because of the pxn-2 bacteria, since this is the only variable that changed. The direct comparison made by the 2-sample T test allowed us to isolate the variable, the pxn-2 bacteria, and establish that the data was statistically significant. We can confidently reject the null hypothesis and state that the unc-70 worms behaved differently due to the pxn-2 bacteria, not chance. It should be noted that the null hypothesis of the 2-sample T test could not be rejected without first performing the ANOVA test.
and rejecting the ANOVA null hypothesis. By rejecting both hypotheses, we can assert that the behavior of the unc-70 worms in pxn-2 bacteria differs from that of the other worms through a changed variable rather than random chance. Through statistical analysis, we were able to convincingly demonstrate that the pxn-2 knockdown (changed variable) caused inhibited axon regeneration in the unc-70 worms in pxn-2 bacteria (different behavior).

In the unc-70 worms in pxn-2 bacteria, the majority of incomplete commissures stopped abruptly halfway between the ventral and dorsal nerve cords. Also, the unc-70 worms appeared to have more growth cones on their ventral nerve cord than the wild type worms. Lastly, on the worm plates that contained unc-70 worms in pxn-2 bacteria, very few adult stage worms were observed, indicating that the unc-70 worms in pxn-2 bacteria had trouble surviving to adulthood.

**Discussion**

The data from the unc-70 worms in pxn-2 bacteria strongly suggests that the presence of pxn-2 is important for axon regeneration, since the suppression of pxn-2 inhibited axon regeneration. As the function of peroxidasins is unknown, the cause of the inhibited axon regrowth is also unclear. In order for axon regeneration to occur, the environment in the ECM must be optimal. As pxn-2 is likely to interact with the axon regeneration environment, we will explore three hypotheses related to its potential functionality within the ECM.

Studies on the ECM of C. elegans have found that type IV collagen is responsible for the scaffolding of the basement membrane in C. elegans, which is a thin layer of the ECM that attaches the muscle to the epidermis. Another study shows that type XVIII collagen in the basement membrane of C. elegans affects axon guidance, indicating that basement membranes play a role in axonal guidance cues. Finally, type IV collagen is believed to be the substrate for peroxidasins, since the absence of peroxidasins caused muscle detachment in C. elegans. If peroxidasins are located in the basement membrane, the pxn-2 knockdown could have prevented the axons from integrating into the muscles by impeding the scaffolding process of the basement membrane. Without proper scaffolding, it would be extremely difficult for an axon to find its way from the ventral nerve cord to the dorsal nerve cord, especially if the guidance cues were not aligned with the basement membrane scaffold. Additionally, the muscle detachment explains the lack of adults on the worm plates containing unc-70 worms in pxn-2 bacteria, since the worms would not survive long without their muscles adhered to the epidermis by the basement membrane.

The next two hypotheses discuss axon guidance cues, specifically chemotaxis. In C. elegans, axons of Gaba-ergic DD motor neurons are guided by secreted proteins called chemoattractants and chemorepellents. These axons are usually repelled away from the ventral nerve cord by chemorepellents and then attracted towards the dorsal nerve cord by chemoattractants. It is known that many of the proteins that act as guidance cues reside or are secreted in the ECM, making the interaction between axonal growth cones and the ECM extremely important for successful axon regrowth. Since the qualitative data in this experiment indicates that the incomplete axons did make it away from the ventral nerve cord but did not travel all the way to the dorsal nerve cord, it seems that the chemoattractant gradient was disturbed. A functioning chemoattractant gradient has a high concentration of chemoattractants near the dorsal nerve cord that decreases in concentration and results in a low concentration of chemoattractants near the ventral nerve cord. This way, the axon is pulled towards the higher concentration of chemoattractants, guiding the axon to the dorsal nerve cord. The unc-6 netrin is an example of an ECM protein that produces chemoattractants and is located near the dorsal nerve cord. If the chemoattractant gradient is disrupted, the guidance cues are unclear, and axon regeneration fails. Since the incomplete commissures in the unc-70 worms in pxn-2 bacteria seemed to have trouble attracting to the dorsal nerve cord, pxn-2 knockdown may have disrupted the chemoattractant gradient.

The third hypothesis deals with another aspect of chemotaxis, the chemorepellent gradient. Through quantitative analysis, both groups of unc-70 worms appeared to be missing commissures, as their total average number of commissures did not match the total average number of commissures of the wild type worms. Through qualitative analysis, additional growth cones were observed near the ventral nerve cord in the unc-70 worms. Since growth cones are the foremost part of a regenerating axon, it is possible that the additional growth cones are the foremost parts of the missing commissures attempting to regrow. However, since few growth cones were observed in the middle of the unc-70 worms, it can be assumed that the growth cones were having trouble leaving the ventral nerve cord. If this is the case, the chemorepellent gradient may be disrupted as well, because it is the job of chemorepellents to guide regenerating axons away from the ventral nerve cord. The unc-5 netrin is an example of an ECM protein that produces chemoattractants and is located near the ventral nerve cord. The additional growth cones observed in the unc-70 worms may also be attributed to pxn-2 knockdown, which could
have disturbed the chemorepellent gradient.

These hypotheses suggest three different functions of peroxidasins, but all three imply that peroxidasins help provide an optimal environment in the ECM for axon regeneration. Quantitative data from this experiment strongly indicates that pxn-2 is an essential element of successful axon regrowth. It is through qualitative data that we are able to make reasonable hypotheses as to the cause of the observed inhibition of axon regeneration. These hypotheses are more like shrewd conjectures than conclusive explanations, and as the box plots indicate, these results could always use more supporting data. However, we feel that the data provided is a fairly accurate representation of the populations and behaviors of the four worm groups.

In future studies, we would like to quantify some of the qualitative observations that these hypotheses were based upon. We focused on commissure counts as the most effective way to analyze the data, but growth cones could also be counted and distance of axons from the ventral nerve cord could be measured. This data may be able to clarify some of the unanswered questions in the three hypotheses from this experiment. For example, if type IV collagen is the substrate for pxn-2, then some of the effects of the pxn-2 knockdown should be unrelated to axon regeneration. If this is the case, it is odd that the wild type worms behaved almost identically, since the wild type worms should exhibit pxn-2 knockdown effects. Another question is whether the incomplete commissures were caused by a problem with the chemoattractant or chemorepellent gradient,

Figure 7. This box plot represents the data set of complete commissures. The gray boxes indicate the Inner Quartile Range (IQR), which is the middle 50% of the data, and the median lines are within the IQR. The medians are 15, 15, 10, and 6 for the wild type worms in control and pxn-2 bacteria and unc-70 worms in control and pxn-2 bacteria, respectively. The lines extending from the IQR show the range of data, not including outliers. The gray dots represent outliers (an outlier is any data point greater than the 3rd quartile value plus 1.5 times the IQR or less than the 1st quartile value minus 1.5 times the IQR).

Figure 8. This box plot represents the data set of incomplete commissures. The gray boxes indicate the Inner Quartile Range (IQR), which is the middle 50% of the data, and the median lines are within the IQR. The medians are 2 and 5 for the unc-70 worms in control and pxn-2 bacteria, respectively. The lines extending from the IQR show the range of data, not including outliers. The gray dots represent outliers (an outlier is any data point greater than the 3rd quartile value plus 1.5 times the IQR or less than the 1st quartile value minus 1.5 times the IQR).

or both.

Future studies of peroxidasins should explore the role of pxn-2 in other species with homologous genes, especially in model organisms like Drosophila. This experiment could be redesigned to focus on quantifying some of the qualitative data in C. elegans, but the importance of pxn-2 in axon regeneration has been demonstrated. Instead, a more effective research tactic would be to identify the function of peroxidasins in other creatures in order to corroborate or contradict the hypotheses from this experiment. Also, the location of peroxidasins in the basement membrane of the ECM is not yet certain, so manipulating peroxidasins in other species may help isolate the location and substrate of pxn-2 in C. elegans and other species.

To explain the results of this experiment, we hypothesized that the pxn-2 knockdown inhibited axon regeneration through its type IV collagen substrate, through the chemoattractant gradient, or through the chemorepellent gradient. We demonstrated that the effect of pxn-2 knockdown is statistically significant in inhibiting axon growth in C. elegans, indicating that pxn-2 plays an important role in axon regeneration. The eventual goal of this research is to identify homologous genes with Homo sapiens to try and induce axon regeneration of the central nervous system in humans. Since peroxidasins have homologous genes in humans, the results of this experiment may be significant in the search for a cure to paralysis. However, the function of the elusive peroxidasin protein family still remains a mystery to be solved by further research.
References


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