Optic nerve regeneration in the mouse is a complex trait modulated by genetic background

Jiaxing Wang,[1,2] Ying Li,[2] Rebecca King,[2] Felix L. Struebing,[2] Eldon E. Geisert[2]

[1]*Department of Ophthalmology, Tianjin Medical University General Hospital, Tianjin, China;* [2]*Department of Ophthalmology, Emory University, Atlanta, GA*

Purpose: The present study is designed to identify the influences of genetic background on optic nerve regeneration using the two parental strains (C57BL/6J and DBA/2J) and seven BXD recombinant inbred mouse strains.

Methods: To study regeneration in the optic nerve, *Pten* was knocked down in the retinal ganglion cells using adenoassociated virus (AAV) delivery of shRNA, and a mild inflammatory response was induced with an intravitreal injection of zymosan with CPT-cAMP. The axons of the retinal ganglion cells were damaged by optic nerve crush (ONC). Following a 12-day survival period, regenerating axons were labeled by cholera toxin B, and 2 days later, the regenerating axons within the optic nerve were examined. The number of axons at 0.5 mm and 1 mm from the crush site were counted. In addition, we measured the distance that five axons had grown down the nerve and the longest distance a single axon reached.

Results: The analysis revealed a considerable amount of differential axonal regeneration across the seven BXD strains and the parental strains. There was a statistically significant difference (p=0.014 Mann–Whitney U test) in the regenerative capacity in the number of axons reaching 0.5 mm from a low of 236.1±24.4 axons in the BXD102 mice to a high of 759.8±79.2 axons in the BXD29 mice. There were also statistically significant differences (p=0.014 Mann–Whitney U test) in the distance axons traveled. Looking at a minimum of five axons, the shortest distance was 787.2±46.5 µm in the BXD102 mice, and the maximum distance was 2025.5±223.3 µm in the BXD29 mice.

Conclusions: Differences in genetic background can have a profound effect on axonal regeneration causing a threefold increase in the number of regenerating axons at 0.5 mm from the crush site and a 2.5-fold increase in the distance traveled by at least five axons in the damaged optic nerve.

Over the last decade, considerable advances have been made in approaches for inducing regeneration of retinal ganglion cell (RGC) axons through the optic nerve [1-5]. The regeneration and survival of RGCs are influenced by interactions between multiple cellular processes (for a review, see [5-7]). The number of genes and molecular pathways that modulate the regenerative response in the mammalian optic nerve reveals that induced axonal regeneration (or the lack of regeneration in the normal adult central nervous system) is a complex trait [1,2,8-11]. Complex traits are controlled by multiple genomic elements; some are associated with specific molecular functions, and others are believed to be associated with more generalized cellular functions [12-14]. This complexity of axonal regeneration can be predicted because we know that successful regeneration involves multiple cellular processes. The first process is the survival of the injured retinal ganglion cell involving modulating apoptosis [15,16], autophagy [1], and response to growth factors [11,17-19]. The second process necessary for axonal regeneration to occur is the growth of the axon itself down the optic nerve. This includes distinct pathways associated with the axon growth program [20]. The third series of events may be directly related to cellular elements that inhibit axonal growth in the adult central nervous system that are glial in origin, involving astrocytes [21,22], oligodendrocytes [10], or the glial scar [21,22]. One approach in studying regeneration is to use inbred mouse strains, identifying strains and genetic backgrounds that facilitate axonal regeneration. Omura et al. [8] tested nine inbred strains and found that one strain (CAST/Ei) was capable of a considerable amount of axon regeneration on inhibitory substrates in tissue culture. The CAST/Ei strain also demonstrated a relative robust regeneration in vivo compared to the C57BL/6J strain. Our goal in the present study is to take a similar systems biology approach to the study of optic nerve regeneration.

Our working hypothesis is that current regeneration treatments can be influenced by the genetic background and within that genetic background are specific genomic elements that can be identified. Our group has used a systems biology approach working with the BXD recombinant inbred (RI) strains of mice to define genomic elements affecting the response of the retina to optic nerve damage [23] and to blast injury [24]. The power of the BXD strain set derives from

Correspondence to: Eldon E. Geisert, Emory University, 1365B Clifton Road NE; Atlanta GA 30322; Phone: (404) 778-4239; FAX: (404) 778-2231; email: egeiser@emory.edu

the shuffled genomes of the parental strains (C57BL/6J and DBA/2J mice). Both parental strains are fully sequenced, and there are more than 4.8 million known single nucleotide polymorphisms (SNPs), deletions, and insertions between the strains. In the first 102 BXD strains, there are more than 7,000 breakpoints in the genomes between the parental strains. All of the BXD strains are fully mapped. This allows rapid mapping of phenotypic data on genomic elements to define loci that modulate the phenotype in a quantitative trait analysis [25,26]. All of these information and powerful bioinformatic tools are available on the GeneNetwork website and are used to define the complex genetics underlying induced regeneration in the optic nerve.

We use the BXD recombinant inbred strains to examine the regeneration response 14 days after optic nerve crush in mice in which *phosphatase and tensin homolog* (*Pten*) was knocked down and zymosan and CPT-cAMP were injected into the vitreous chamber [1,3]. Regenerative response is determined by defining the number of axons regenerating, as well as the distance these axons have traveled.

METHODS

Mice: Nine strains, including seven BXD recombinant inbred strains and their parental strains, C57BL/6J and DBA/2J, were used in this study. All of the mice were 60–70 days of age at the time of initial treatment (Appendix 1). The mice were housed in a pathogen-free facility at Emory University, maintained on a 12 h:12 h light-dark cycle, and provided with food and water ad libitum. All procedures involving animals were approved by the Animal Care and Use Committee of Emory University and were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Controls were run with the C57BL/6J (n=6) and DBA/2J (n=6) mice strains. For the regeneration studies, we examined axon growth in the parental strains, C57BL/6J (n=5) and DBA/2J (n=8), along with seven BXD strains: BXD11 (n=5), BXD29-*Tlr4*lps-2J/J (n=4), BXD31 (n=4), BXD38 (n=4), BXD40 (n=9), BXD75 (n=5), and BXD102 (n=5).

Surgery: The optic nerve regeneration protocol developed by others [1,3,4] was used to induce regeneration after optic nerve crush (ONC). The treatment included knocking down of *Pten* using adenoassociated virus-shPTEN-green fluorescent protein (AAV-shPTEN-GFP) and intravitreal injection of zymosan plus CPT-cAMP. We followed a similar protocol with minor modifications. One is that we used AAV-shPTEN-GFP (*Pten* short hairpin RNA-GFP packaged into AAV2 backbone constructs, titer = 1.5×10^{12} vg/ml) to knock down *Pten* instead of Cre recombinase–mediated knockout in *Pten*-floxed mice. The shRNA target sequence is

5′-AGG TGA AGA TAT ATT CCT CCA A-3′ as described by Zukor et al. [27]. The immunostaining also proved efficient suppression of *Pten* expression in the retina ganglion cells by this *Pten* shRNA (Appendix 2). Two weeks before ONC, the mice were deeply anesthetized with 15 mg/kg of xylazine and 100 mg/kg of ketamine and intravitreal injection of 2 µl of AAV-shPTEN-GFP. Optic nerve crush was performed as described by Templeton and Geisert [28]. Briefly, the mice were deeply anesthetized with a mixture of 15 mg/kg of xylazine and 100 mg/kg of ketamine. Under the binocular operating scope, a small incision was made in the conjunctiva, and then the optic nerve was visualized and crushed 1 mm behind the eye with Dumont N7 angled crossover tweezers for 5 s, avoiding injury to the ophthalmic artery. Immediately following ONC, zymosan (Sigma, St. Louis, MO; Z4250, Lot # BCBQ8437V) along with the cAMP analog CPT-cAMP (Sigma, C3912, Lot # SLBH5204V; total volume 2 µl) were injected into the vitreous to induce an inflammatory response and augment regeneration. Mice were given buprenorphine SQ at 0.5 mg/kg immediately following the optic nerve crush. When the mice were fully recovered, they were returned to a clean cage and monitored for 3 days post-op. Mice showing signs of pain or distress were euthanized via cervical dislocation after being deeply anesthetized with ketamine/xylazine at 100 mg/kg and 15 mg/kg, respectively. Twelve days after ONC (2 days before euthanasia) the animals were deeply anesthetized, and Alexa Fluor® 647-conjugated Cholera Toxin B (CTB; ThermoFisher, Waltham, MA; C34778) was injected into the vitreous for retrograde labeling of the regenerated axons. All the intravitreal injections and optic nerve crushes were performed by one well-trained postdoctoral fellow to avoid technical variation during the surgical procedure. At 14 days after ONC, the mice were deeply anesthetized with a mixture of 15 mg/kg of xylazine and 100 mg/kg of ketamine and perfused through the heart with PBS (Diluted to 1x from 10x, Corning, Manassas, VA, pH 7.3) followed by 4% paraformaldehyde in phosphate buffer (pH 7.3).

Preparation of the optic nerve: Optic nerves along with the optic chiasm and brains were dissected and post-fixed in 4% paraformaldehyde in phosphate buffer overnight. The optic nerve was cleared with FocusClear™ (CelExplorer, Hsinchu, Taiwan) for up to 4 h until totally transparent. A small chamber was built on the slide to provide enough space for the whole nerve thickness and to keep the nerve from being damaged from flattening. The optic nerve was then mounted in the chamber using MountClear™ (CelExplorer), and the slides were coverslipped. FocusClear has been used to clear brain tissue for whole brain imaging [29], as well as clearing of the optic nerve of transgenic zebrafish to observe axon regeneration [30]. FocusClear allows us to scan the
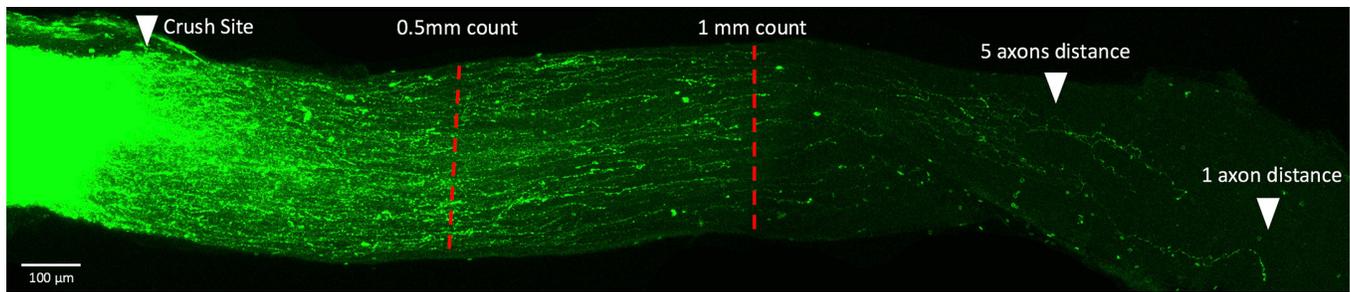
Figure 1. The regenerating axons in the optic nerve 14 days after optic nerve crush. We indicated the regions of the nerve where axons were counted, as well as the distance that five axons or one axon regenerated down the nerve. Scale bar = 100 μm.

whole thickness of the optic nerve for better understanding of the status of axon regeneration. It provided clear imaging of regenerated axons from the optical slices scanned by confocal microscope for counting (Appendix 3). FocusClear also allowed us to determine the longest five axons or single axon growth along the nerve from the z-stack of the whole nerve (Figure 1).

Quantitation of axon regeneration: Cleared optic nerves were examined with a confocal microscope by scanning through individual optical slices. Pseudocolor green was used for the CTB-labeled axons in all the optic nerve images of this study for clear visual observation. Stacked images were taken at 10 μm increments, a total of 20–50 optical slices for each optic nerve.

The number of CTB-labeled axons at 0.5 mm from the crush site were counted in at least six sections per case and calculated with the equation

$$\sum ad = \pi r^2 \cdot [average \ axons/mm]/t$$

as described by Leon et al. in 2000 [31]. In this formula, the cross-sectional width of the nerve was measured at the point at which the counts were taken and was used to calculate the number of axons per millimeter of nerve width. The total number of axons extending distance d in a nerve having a radius of r (half of the biggest width of all optic sections) was estimated by summing all sections. The virtual thickness of an optical slice observed with the confocal microscope was calculated using the formula

$$dz \cong \frac{0.64 \times \lambda exc}{n - \sqrt{n^2 - NA^2}}$$

We determined that the thickness of the optical section was 6 μm where the refractive index (n) was 1.517, the numerical aperture (Na) was 0.45, and the excitation wavelength was 637 nm. As the optical section was 6 μm and the spacing between the optical sections was 10 μm, single axons were

not counted multiple times. For quantifying the number of axons at 1 mm from the crush site, as there are very few axons observed in some strains, we used direct counts of axons as the measure of regeneration. We also measured the distance that five axons had grown down the nerve and the longest distance a single axon reached for each nerve from the z-stack image of the whole nerve.

Transfection efficiency of AAV-shPTEN-GFP: BXD102 mice (n=4) and BXD29 mice (n=4) were deeply anesthetized with a mixture of 15 mg/kg of xylazine and 100 mg/kg of ketamine and injected with 2 μl of AAV-shPTEN-GFP into the left eye. Two weeks later, they were deeply anesthetized as described above and perfused through the heart with saline followed by 4% paraformaldehyde in phosphate buffer (pH 7.3). For the retinal flat mounts, the retinas were removed from the globe and rinsed in PBS with 1% Triton X-100, blocked in 5% bovine serum albumin (BSA) for 1 h at room temperature, and placed in primary antibodies RBPMS (Millipore, Burlington, MA; Cat. # ABN1376) at 1:1000 and GFP (Novus Biologicals, Littleton, CO; Cat. # NB100–1770) at 1:1,000 at 4 °C overnight. The retinas were rinsed with PBS and placed in secondary antibodies (Anti-Goat Immunoglobulin G [IgG] (H⁺L) CFᵀᴹ 488A, Sigma, Cat. # SAB4600032 and Alexa-Fluor 594 AffiniPure Donkey Anti-Guinea (Jackson Immunoresearch, West Grove, PA, Cat. #706–585–148) at 1:1,000 for 1 h at room temperature. After three washes for 15 min each, the retinas were flat mounted and coverslipped using Fluoromount-G (Southern Biotech, Birmingham, AL; Cat. # 0100–01) as the mounting medium. Four confocal images were taken in each quadrant at 2 mm from the optic nerve of each retina. Four retinas from four mice of each strain were included. Cell numbers were determined manually by using the cell counter in ImageJ. RBPMS was used as a marker to label the total number of RGCs [32,33]. Transfection efficiency are calculated as the number of AAV-transfected RGCs (GFP-positive RBPMS-positive cells) divided by total number of RGCs (RBPMS-positive cells).

TABLE 1. GENETIC CHARACTERISTICS OF GENES THAT ARE KNOWN TO AFFECT OPTIC NERVE REGENERATION BETWEEN C57BL/6J AND DBA/2J MICE.	
Gene with cis-QTL	*Fgf2* [41]
Genes with Non-synonymous SNPs	*Mapk10* [42], *Rtn4* [43,44], *Ctgf* [11], *Tlr2* [45], *Rock1* [46], *Rock2* [46,47], *Clec7a* [45], *Csf2* [48]
Other investigated genes (Genes that are found to have neither cis-QTL nor Non-synonymous SNPs)	*Braf* [49,50], *Bcl2* [9], *Myc* [51], *Cntf* [52], *Dpysl2* [53], *Ecel1* [54], *Map3k12* [55], *Egfr* [17], *Gsk3b* [56,57], *Hhex* [58], *Hnrnpk* [59], *Spp1* [60], *Stat3* [2,61,62], *Klf4* [2,62,63], *Klf6* [2,63,64], *Klf7* [2,63,64], *Klf9* [2,63], *Lif* [52,65], *Rtn4r* [10,66,67], *Ntn1* [68,69], *Pten* [1], *Ptprs* [70], *Rhoa* [71-73], *Cxcl12* [74], *Set* [75], *Socs3* [37,76], *Sox11* [5,77], *Tet1* [78], *Tet3* [78], *Wnt10b* [79], *Slc30a3* [80], *Bag1* [81] *Inhba* [8]

Bioinformatic analysis of known regeneration genes in the BXD strain set: By searching the literature, we generated a list of genes that are known to have effects on optic nerve regeneration either directly or indirectly (Table 1). All of the genes were examined for high likelihood ratio statistic (LRS) scores and cis-quantitative trait loci (cis-QTLs) using the GeneNetwork database. The genes were then put into the Single Nucleotide Polymorphism browser of GeneNetwork, as well as the UCSC Genome Browser (Mouse, GRCm38/mm10) to identify nonsynonymous SNPs between C57BL/6J and DBA/2J. All the identified rsIDs of nonsynonymous SNPs were then put into Ensembl for Sorting Intolerant From Tolerant (SIFT) analysis [34] to predict whether the SNP affects protein function.

Statistical analysis: Data are presented as mean ± standard error of the mean (SEM). Differences in axon counts, regeneration distance, and transfection efficiency were analyzed with the Mann–Whitney U test using SPSS Statistics package 24.0 (SPSS, IBM, Chicago, IL). A p value of less than 0.05 was considered statistically significant.

RESULTS

Genetic background modulates axon regeneration in RGCs: In the present study, we examined the effects of genetic background on the regenerative response of retinal ganglion cells. The BXD recombinant inbred strains were chosen as the genetic reference panel due to distinct advantages these strains have to offer. The first advantage is that more than 80 well-characterized strains of mice are available through Jackson Laboratories. The BXD RI strain set is considerably larger than any mouse RI strain set, allowing for submegabase mapping resolution. The second benefit of the BXD strains is the large microarray data sets specifically for the eye (HEIMED database [14], HEI Retina Database [35], Optic Nerve Crush Database [23], and the Department of Defense (DoD) Normal Retina Database [36]), as well as the numerous ocular phenotypes RGC numbers, intraocular pressure (IOP), eye size, retinal area, etc. [24]. GeneNetwork also offers an array of highly interactive series of bioinformatic tools that aid in the analysis of data generated with the BXD strains.

Regeneration of axons in the optic nerve was examined in nine strains of mice: the two parental strains (C57BL/6J and DBA/2J) and seven BXD strains (BXD11, BXD29, BXD31, BXD38, BXD40, BXD75, and BXD102; Figure 2). As an internal control, we examined the ability of untreated retinas to regenerate following optic nerve crush in the C57BL/6J mice and the DBA/2J mice (Figure 2). For all mice, axonal regeneration was evaluated 14 days following optic nerve injury. In the two strains of the control group (C57BL/6J and DBA/2J), there was no detectable axonal regeneration; while in all of the strains receiving the regeneration treatment there was a statistically significant regenerative response in the number of axons counted at 0.5 mm and 1 mm (Figure 3), as well in the distance the axons traveled (Figure 4). In the parental strains, the differences between the treatment group and the control group are statistically significant (p<0.01 for the C57BL/6J and DBA/2J mice). These data demonstrate that the regeneration treatment, knocking down *Pten* and inducing mild inflammation by injecting zymosan and CPT-cAMP, produces considerably more regenerating axons than observed in the control animals that did not receive treatment.

The number of axons at 0.5 mm and 1 mm distal to the crush site is an estimate of the influence of genetic background on the total regenerative effect of the treatment. As can be seen in Figure 2 and Figure 3, there is a considerable difference between the strains in the total number of axons reaching 0.5 mm and 1.0 mm. The strain with the least number of axons in both cases was BXD102. At both distances from the crush site, the strain with the greatest number of axons was BXD29. The difference was statistically significant (p=0.014, Mann–Whitney U test) in the number of regenerated axons reaching 0.5 mm (from a low of 236.1±24.40 axons in the BXD102 mice to a high of 759.8±79.20 axons in the BXD29 mice, a 3.2-fold difference) from the crush site. There was also a statistically significant difference (p=0.007, Mann–Whitney U test, a 12.6-fold difference) in the number
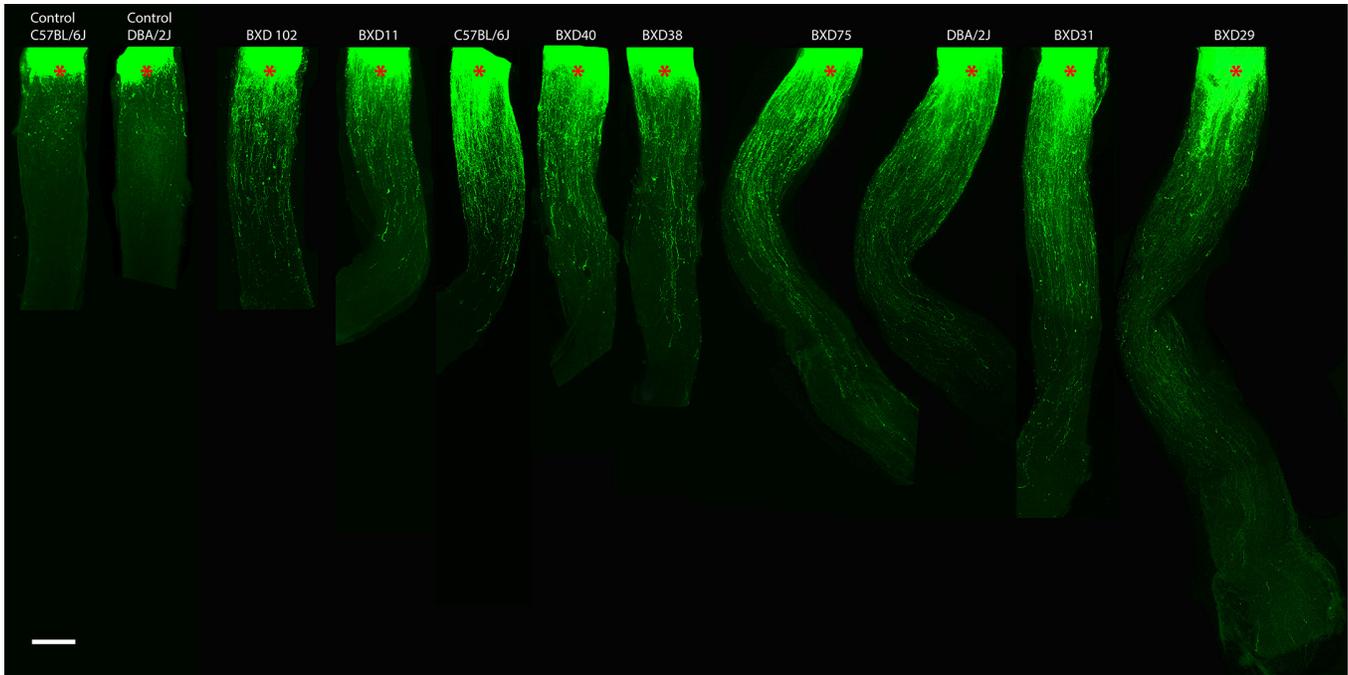
Figure 2. Genetic background affects regenerating axons in the optic nerve following optic nerve crush. The figure is a series of photomicrographs from 11 optic nerves selected from nine strains of mice. The first two images on the far left are from control mice that did not receive the regeneration treatment before optic nerve crush (Control C57BL/6J and Control DBA/2J). All of the remaining nerves were from animals in which *Pten* was knocked down and a mild inflammatory response was induced. The strain with the least regeneration was BXD102, and the strain with the greatest regeneration was BXD29. Red asterisks represent the crush site. Scale bar = 200 μm.

of axons at 1 mm, from a low of 1.00±0.00 axons in the BXD102 mice to a high of 12.6±0.60 axons in the BXD29 mice. The two strains that displayed the least and most robust (BXD102 and BXD29) regenerative response are illustrated in Figure 5.

The total length of a regenerating axon was also measured. This measure may provide an estimate of the rate at which the axon can grow down the injured optic nerve. When we examined axon length, there was also a clear difference in growth across the BXD strains (Figure 2 and
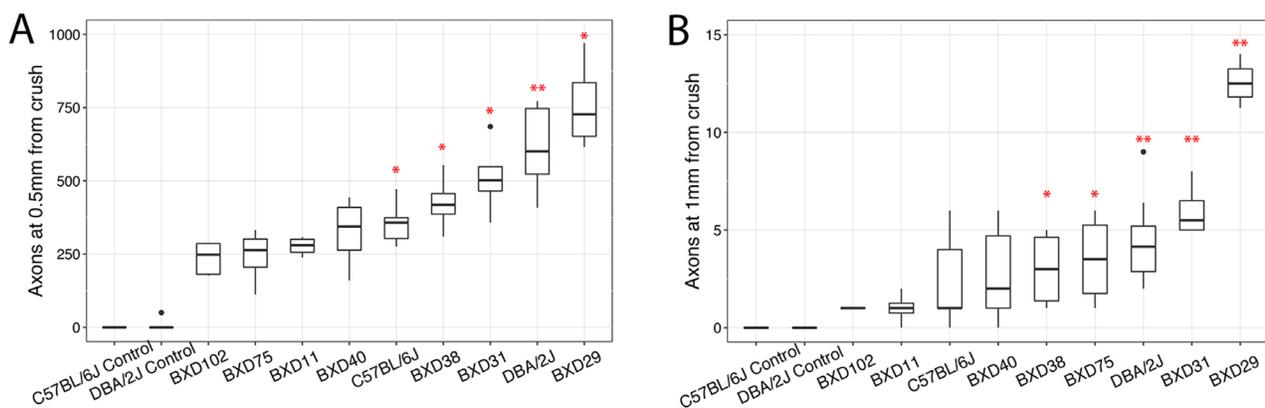

Figure 3. The differences in the number of regenerating axons in BXD strains. The number of axons at 0.5 mm (**A**) and 1 mm (**B**) from the crush site in two control strains (DBA/2J and C57BL/6J untreated mice) and in nine strains treated with the regeneration protocol. Boxplots show median, 25th and 75th percentile, maximum, and minimum values for each BXD recombinant inbredstrain. Black dots: outliers. *: $p < 0.05$ when compared with BXD102. **: $p < 0.01$ when compared with BXD102.
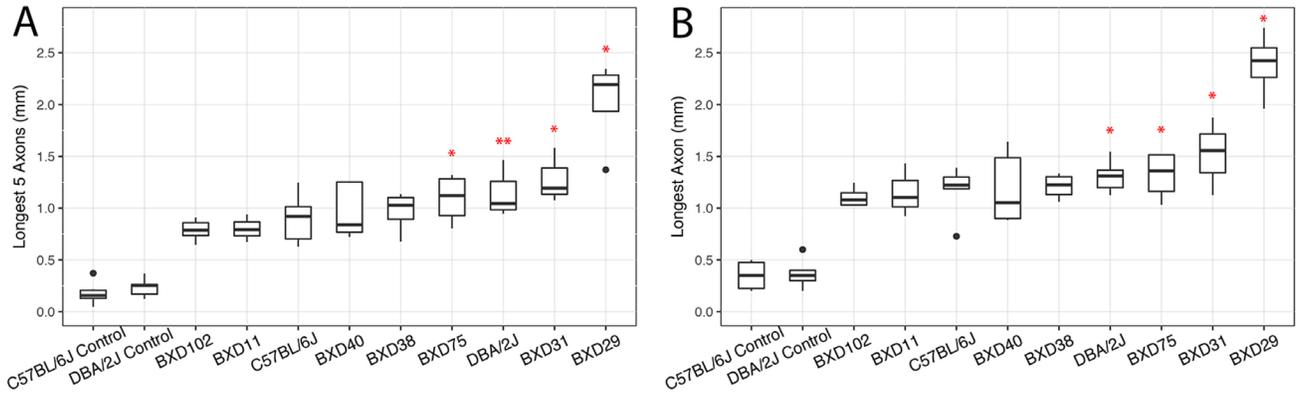
Figure 4. The differential growth of regenerating axons within BXD strains. The longest distance that five axons regenerated (**A**) and the longest regeneration for a single axon (**B**) are shown for the two control strains (DBA/2J and C57BL/6J untreated mice) and in nine strains treated with the regeneration protocol. Boxplots show median, 25th and 75th percentile, maximum, and minimum values for each BXD recombinant inbred strain. Black dots: outliers. *: p<0.05 when compared with BXD102. **: p<0.01 when compared with BXD102.

Figure 4). In the control animals, virtually no regenerating axons were observed. When we examined the distance a minimum of five axons traveled, a statistically significant difference was observed across the BXD strains. The strain with the shortest regenerating five axons was BXD102 with a mean distance of 787.2±46.50 µm, and the strain with the longest group of five axons was BXD29 with a mean distance of 2025.5±223.30 µm (p=0.014, Mann–Whitney U test, a 2.5-fold difference). A similar result was observed when the distance of the longest single axon traveled in the nerve was
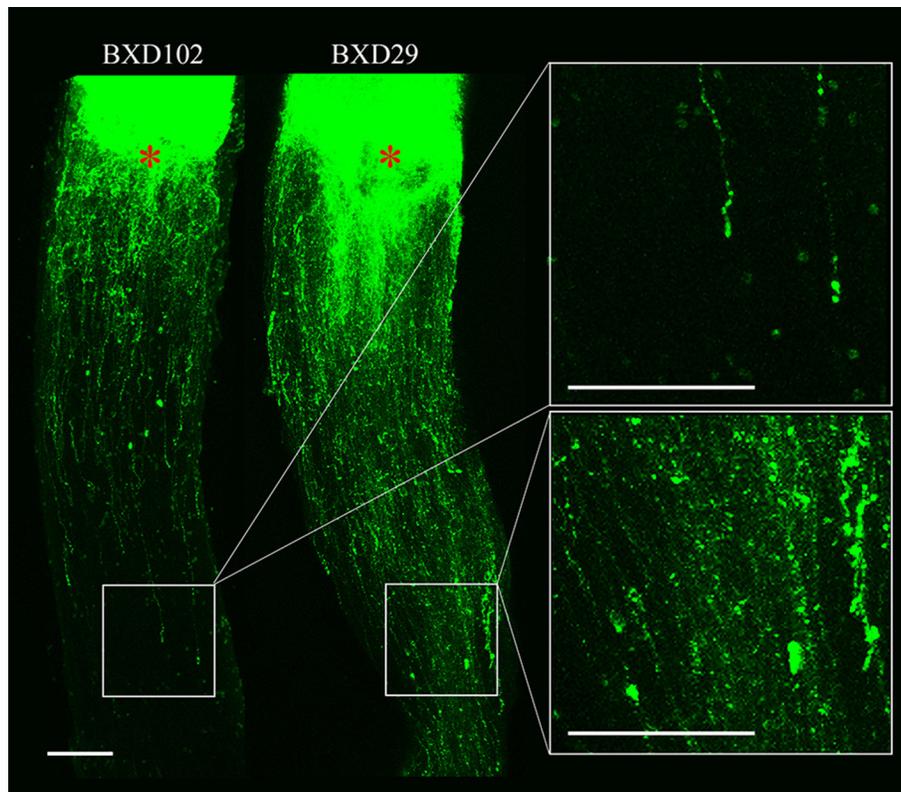

Figure 5. Comparison of regenerated axons in strains with the least regeneration (BXD102) and the greatest regeneration (BXD29). Higher magnification of axons at 1 mm (the boxed region) from the crush site are shown. Scale bar = 100 µm.

examined, with BXD102 having the shortest average distance (1,107±40.60 μm) and BXD29 the longest (2,386.8±162.60 μm, p=0.014, Mann–Whitney U test, a 2.2-fold difference). Thus, the axonal regeneration ability (the number of regenerating axons and the distance traveled) is affected by the genetic backgrounds in the BXD strains with BXD102 having the least regenerative and BXD29 having the most robust regenerative response. These data revealed that genetic background can have a striking effect on the regenerative capacity of axons within the optic nerve.

Transfection efficiency of AAV-shPTEN-GFP: One possible explanation for the difference in axonal regeneration is differential transfection of the retinas from strain to strain by the AAV-shPTEN-GFP vector. To control for this possibility, we examined the transfection efficiency and the level of *Pten* knockdown in the strain with the most robust axon regeneration (BXD29) and the strain with the least axon regeneration (BXD102). There was no statistically significant difference between the two strains. For the BXD29 strain (n=4), the mean transfection rate was 51.6%±1.30%, and for the BXD102 strain (n=4), the mean transfection rate was 50.8%±1.40% (Figure 6), indicating that the difference in the regeneration response is not due to different transfection efficiency.

Potential contribution of known genes affecting axon regeneration in BXD strains: Previous studies have identified several genes that affect the ability of axons to regenerate in the injured optic nerve (Table 1). Using the bioinformatic tools on GeneNetwork, it is possible to define any of the regeneration-associated genes that are either differentially expressed forming a cis-QTL in the BXD strains or have nonsynonymous SNPs between C57BL/6J and DBA/2J mice.

The cis-QTL is a QTL that maps to the location of the gene that produces the mRNA or protein. The LRS score is used to represent the association or linkage between differences in traits and differences in particular genotype markers or specific genes. Although a statistically significant cutoff can be determined only through permutation tests, LRS scores of greater than 17 usually approximate the statistical significance threshold of a p value of less than 0.05 and are worthy of attention [14]. If a cis-QTL has a high LRS score, it is considered that this genetic locus is strongly linked to a certain phenotype and is able to influence the phenotype by regulating this locus. In other words, change in the expression level of this gene will have a higher chance to alter the phenotype, which, in the present case, is the axonal regeneration. In this process, two regeneration-associated genes were identified with cis-QTLs, *Fgf2* and *Klf9* (Table 1). Only one of these cis-QTLs, *Fgf2*, is valid. The other, *Klf9,* contained a difference in the genetic sequences between C57BL/6J and

DBA/2J mice at the exact site where the microarray probe binds. This difference in sequence will lead to differential binding of the probe and a false positive LRS score. Thus, there was one cis-QTL (*Fgf2*) present in the BXD strains that could potentially affect the regenerative response.

We also examined the BXD strains to define genes with nonsynonymous SNPs. A nonsynonymous SNP between the parent strains (C57BL/6J and DBA/2J) is potentially able to alter the protein structure and function, ultimately leading to the different phenotype. The BXD strains that inherited different alleles may also have different phenotypes. There were eight genes (*Mapk10, Rtn4, Ctgf, Tlr2, Rock1, Rock2, Clec7a,* and *Csf2*) with nonsynonymous SNPs between C57BL/6J and DBA/2J. The SIFT analysis [34] revealed that only two of the eight genes, *Mapk10* (JNK3) and *Rtn4* (NOGO), had SNPs that were predicted to likely affect protein structure/function (rs36844177 in *Mapk10,* SIFT=0.01 and rs29465940 in *Rtn4*, SIFT=0.03). Thus, in the BXD strain set, only three genes known to be associated with axonal regeneration, *Fgf2, Mapk10* (JNK3), and *Rtn4* (NOGO), are actively different between C57BL/6J and DBA/2J mice and potentially contribute to the different response of axonal regeneration across the BXD strains.

DISCUSSION

Over the past several years, advances in optic nerve axon regeneration have taken what was once thought of as an unachievable goal to the point where axonal regrowth after injury is a reality. Several different protocols are being used to promote axonal regeneration [2,5,37]. In the present study, we chose a popular protocol developed by others [3,4] that involves knocking down *Pten* and causing a mild inflammatory response. The BXD recombinant strains are ideal for testing the effects of genetic background with the protocol of knocking down *Pten*, for there are no statistically significant differences in *Pten* between the C57BL/6J strain and the DBA/2J strain. There are no nonsynonymous SNPs found in the *Pten* gene between C57BL/6J and DBA/2J mice. Furthermore, there is a similar level of expression of *Pten* mRNA across all of the BXD strains in the DoD normal retina data sets housed on GeneNetwork. The injection of zymosan is believed to involve the activation of an inflammatory response and activation of macrophages [38], stimulating the release of oncomodulin [39]. When we examined the BXD RI strains, there was no statistically significant difference in the levels of oncomodulin message, and there were no nonsynonymous SNPs within the gene. That being said, we do know that there is a considerable immune network in the retina of the BXD RI strains and that this network is

activated by optic nerve crush [23] and blast injury [24]. We also examined transfection efficiency in two strains (BXD29 and BXD102) that respond differently to the regeneration treatment. There was no difference in transfection efficiency between these two strains. Thus, the difference in the axon regeneration we observed between the different BXD strains cannot be explained by the expression levels of *Pten* in the strains or a differential level of transfection by the AAV2 vector. This leaves only the possibility that the difference in axonal regeneration we observed is due to the specific segregation of genomic elements across the BXD strains.

Using the BXD strains, we were able to demonstrate the effect of genetic background on the regenerative capacity of axons in the optic nerve. In all strains tested, the amount of regeneration was considerably greater than that observed in mice that did not receive the *Pten*/zymosan/cAMP treatment. The regeneration responses of C57BL/6J mice we observed were not as strong as described in other studies. A possible reason could be that we used AAV-shPTEN-GFP to knock down *Pten* instead of Cre recombinase–mediated *Pten* knockout. The other factor to be noticed is that the mice we used were older than 60 days at the time of the initial
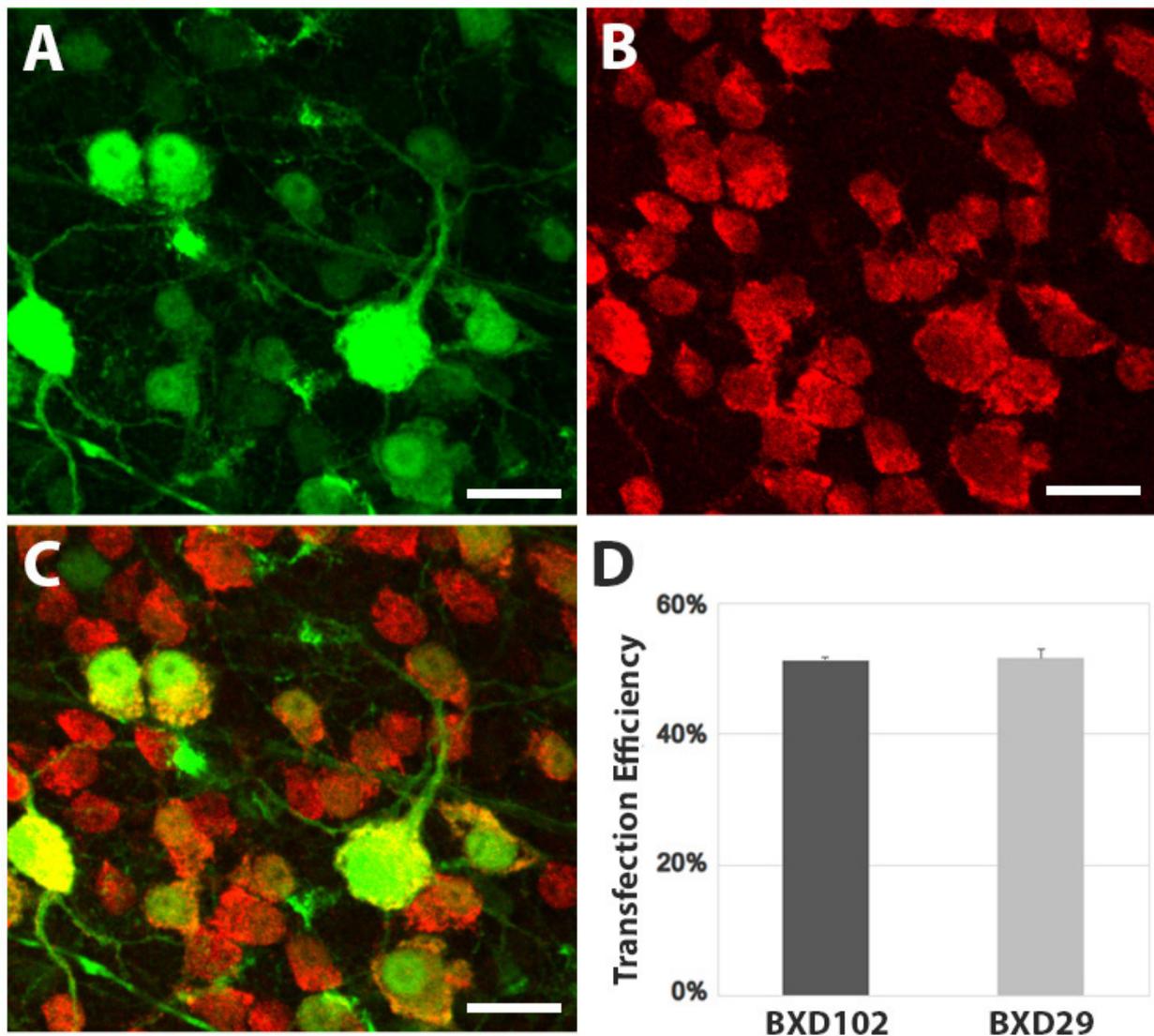

Figure 6. Transfection efficiency of AAV-shPTEN-GFP. **A**: Adenoassociated virus (AAV) –transfected cells are labeled with green fluorescent protein (GFP) in green. **B**: The total number of retinal ganglion cells (RGCs) are labeled by RBPMS in red. **C**: Merged channel is shown. **D**: No statistically significant difference in transfection efficiency was found between BXD102 mice and BXD29 mice. Scale bar = 10 μm.

treatment, much older than reported in other studies [3,4]. This also provides strong evidence that the regeneration response can happen not only in young adult mice but also in older mice. Among the strains treated to promote regeneration, some strains, such as BXD102, showed a modest regenerative response; while other strains, such as BXD29, consistently demonstrated a high number of regenerating axons and axons that traveled longer distances down the injured optic nerve. When the response of the parental strains C57BL/6J and DBA/2J was compared to that of the BXD strains with extreme regenerative responses, there was a clear indication of genetic transgression. If we look at the number of axons at 0.5 mm and 1 mm from the crush site (Figure 3), some BXD strains have fewer axons and some BXD strains have more axons than the parental strains. This difference in regeneration is indicative of genetic transgression. These data reveal that it is not a single genomic locus causing the variability in the regenerative response; for if that were the case, the extremes would be similar to those of the parental strains. This is a clear indication that multiple genomic loci segregate across the BXD strains to affect the regenerative response of the optic nerve axons. Thus, axon regeneration is a complex trait with multiple modulating genomic loci in the BXD strains.

Other studies have looked at multiple inbred strains of mice, identifying an increased regenerative capacity in the neurons of the CAST/Ei strain [8]. In this study, the authors identified *Inhba* as the genetic element contributing to the increased regenerative capacity. Interestingly, neurons from the BXD parental strains (C57BL/6 and DBA/2J mice) were also tested in this tissue culture system, and neurons from these two strains did not grow well on inhibitory substrates. These data indicate that the genomic elements facilitating regeneration in the CAST/Ei strain are not present in the BXD strain set. When we examined *Inhba* in the DoD Normal Retinal database on GeneNetwork, this gene did not vary statistically significantly across the strains, and it did not display a statistically significant QTL. Furthermore, there are no nonsynonymous SNPs in *Inhba* between the C57BL/6J and DBA/2J strains. These data suggest that novel elements segregate across the BXD strain modulating regenerative capacity.

A complex trait could be driven by a handful of protein coding genes, as well as noncoding variants that presumably affect gene regulation [40]. In recent years, multiple genes have been identified to have an impact on axon regeneration (Table 1). It is possible that some or all of these pathways vary in the BXD strains and influence the outcome of the induced regeneration observed in the present study. With the DoD normal retina data sets [36] and the bioinformatic tools hosted on GeneNetwork, we examined all of the known regeneration-related genes to determine whether they were able to modulate the regenerative response across the strains by having either cis-QTLs (differentially expressed genes) or nonsynonymous SNPs that would affect protein function. Of all of the genes known to alter the regenerative response of optic nerve axons, only two were potential candidates for modulating regeneration in the BXD strains. The only cis-QTL in the list of genes was *Fgf2* (LRS=67.8). SNP analysis identified eight genes with nonsynonymous SNPs in the list of regeneration genes (Table 1). All of the nonsynonymous SNPs were examined using a SIFT analysis, and only two SNPs (rs36844177 in *Mapk10*(JNK3) and rs29465940 in *Rtn4*(NOGO)) were predicted to alter protein function. Therefore, three possible genomic elements that could affect regeneration in the BXD strains are *Fgf2* (Chr3, 37.3 Mb), *Mapk10* (Chr5, 102.9 Mb), and *Rtn4* (Chr11, 29.7 Mb). Beyond these genomic elements, we believe that there are still unknown genomic elements that modulate the regenerative response of the axons.

In conclusion, the ability of optic nerve regeneration to respond to injury differs across BXD mouse strains given different genetic backgrounds. Quantitative trait analysis may provide new insights into axon regeneration, and perhaps new loci of novel genes or noncoding elements that are involved in axon regeneration. Ongoing experiments are increasing the number of strains in the experimental data set to define genomic loci that modulate optic nerve regeneration.

APPENDIX 1. SUMMARY OF OPTIC NERVE REGENERATION IN THE BXD STRAINS

To access the data, click or select the words "Appendix 1"

APPENDIX 2.

To determine if *Pten* was knocked down by our AAV treatment, we stained the retina for *Pten* (Red) in retinas injected with the AAV-GFP vector (control) or AAV-shPTEN-GFP to knock down *Pten*. In the control retina, all of the GFP positive cells were also well labeled for *Pten*. In the retinas that received the AAV-shPTEN-GFP, the transfected cells (GFP positive) had low levels of *Pten* staining indicating that the vector had in fact decreased the expression of *Pten*. To access the data, click or select the words "Appendix 2"

APPENDIX 3. SUPPLEMENTAL MOVIE 1.

To access the data, click or select the words "Appendix 3"

ACKNOWLEDGMENTS

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