The BXD Mouse Strains are a Model System for Studying Optic Nerve Regeneration

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Abstract

The present study is designed to identify the influences of genetic background to optic nerve regeneration using the two parental strains C57BL/6J and DBA/2J and 7 BXD recombinant inbred strains. To study regeneration in the optic nerve, *Pten* was knocked down in the retinal ganglion cells using AAV, and a mild inflammatory response was induced by an intravitreal injection of zymosan with CPT-cAMP, and the axons were damaged by optic nerve crush (ONC). Regenerating axons were labeled by Cholera Toxin B and quantified 14 days after ONC. The number of axons at 0.5 mm and 1 mm from the crush site were counted. In addition, we measured the distance that 5 axons had grown down the nerve and the longest distance a single axon reached. Results showed a considerable amount of differential axonal growth across all 9 BXD strains. There was a 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 1487.6 ± 264.9 axons in BXD102 to a high of 4175.8 ± 648.6 axons in BXD29. There were also significant differences (P=0.014 Mann-Whitney U test) in the distance axons traveled, looking at a minimum of 5 axons with the shortest distance was 787.2 ± 46.5μm in BXD102 to a maximum distance of 2025.5 ± 223.3μm in BXD29. These results reveal that genetic background can modulate axonal regeneration and that the BXD strains are a particularly well-suited model system.

INTRODUCTION

Over the last decade, significant advances have been made in approaches to induce regeneration of retinal ganglion cell (RGC) axons through the optic nerve[1-5] . The regeneration and survival of RGCs is influenced by interactions between multiple cellular processes (for 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 CNS) is a complex trait[1, 2, 8-11]. Complex traits are controlled by multiple genomic elements, some of which 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 could be predicted for we know that successful regeneration involves multiple cellular processes. The first is the survival on the injured retinal ganglion cell involving modulating apoptosis[15, 16], autophagy[1] and response to growth factors[11, 17-19]. The second necessity 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 CNS that are glial in origin, involving astrocytes[21, 22], oligodendrocytes[10] or the glial scar[21, 22]. Our goal is to take a systems biology approach to the study of optic nerve regeneration and to treat the process as a complex trait.

Our working hypothesis is that current regeneration treatments can be influenced by 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 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 the shuffled genomes of the parental strains (C57BL/6J mice and the DBA/2J mice). Both of the parental strains are fully sequenced and there are over 4.8 million known single nucleotide polymorphisms, deletions, and insertions between them. In the first 102 BXD strains, there are over 7000 break-points in the genomes between the parental strains. All of the BXD strains are fully mapped. This allows for a rapid mapping of phenotypic data onto genomic elements to define loci modulating the phenotype in a quantitative trait analysis [25, 26]. All of these information and powerful bioinformatic tools are available on the GeneNetwork website

77 (www.genenetwork.org) and are used to define the complex genetics underlying induced
78 regeneration in the optic nerve.
79
80 We use the BXD recombinant inbred strains to examine the regeneration response 14 days after
81 optic nerve crush in mice that have received a previously described[1, 3] regeneration treatment
82 of knocking down *Pten* (phosphatase and tensin homolog) and intravitreal injection of zymosan
83 and CPT-cAMP. Regenerative response is determined by defining the number of axons
84 regenerating as well as the distance these axons have traveled.
85

MATERIALS AND 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 (See supplemental Table 1). The mice were housed in a pathogen-free facility at Emory University, maintained on a 12-hour 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 studies of regeneration, 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(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 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 = $1.5x10^{12}$ vg/ml) to knock down *Pten* instead of cre recombinase-mediated knock-out in *Pten*-floxed mice. The shRNA target sequence is 5'-AGGTGAAGATATATTCCTCCAA-3' as described by Zukor K et al[27]. Our immunostaining also proved an efficient suppression of *Pten* expression in the retina ganglion cells by this *Pten* shRNA (Supplementary Figure 1). Two weeks prior to ONC, the mice were deeply anesthetized with 15 mg/kg of xylazine and 100 mg/kg of ketamine and injected intravitreally with 2µL 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, the optic nerve was visualized and then crushed 1 mm behind the eye with angled crossover tweezers (Dumont N7) for 5 seconds, avoiding injury to the ophthalmic artery. Immediately following ONC, Zymosan (Sigma, Z4250, Lot#BCBQ8437V) along with the cAMP analog CPT-cAMP (Sigma, C3912, Lot#SLBH5204V)

117 (total volume 2μL) were injected into the vitreous to induce an inflammatory response and
118 augment regeneration. The animals were allowed to recover from anesthesia and returned to their
119 cages. Twelve days after ONC (2 days before sacrifice) the animals were deeply anesthetized
120 and Alexa Fluor® 647-conjugated Cholera Toxin B (CTB) (ThermoFisher, C34778) was
121 injected into the vitreous for retrograde labeling of the regenerated axons. All the intravitreal
122 injections and optic nerve crushes were done by one well-trained postdoctoral fellow to avoid
123 technical variation during the surgical procedure. At 14 days after ONC the mice were deeply
124 anesthetized and perfused through the heart with phosphate buffered saline (pH 7.3) followed by
125 4% paraformaldehyde in phosphate buffer (pH 7.3).
126
127 **Preparation of the optic nerve:** Optic nerves along with optic chiasm, and brains were
128 dissected and post fixed in 4% paraformaldehyde in phosphate buffer overnight. The optic nerve
129 was cleared with FocusClear™ (CelExplorer, Hsinchu, Taiwan) for up to 4 hours until totally
130 transparent. A small chamber was built on the slide to provide enough space for the whole nerve
131 thickness and to keep the nerve from being damaged from flattening. The optic nerve was then
132 mounted in the chamber using MountClear™ (CelExplorer, Hsinchu, Taiwan) and the slides
133 were cover-slipped.
134 FocusClear has been used to clear brain tissue for whole brain imaging [29] as well as clearing of
135 the optic nerve of transgenic zebrafish to observe axon regeneration [30]. It allows us to scan the
136 whole thickness of the optic nerve for better understanding of the status of axon regeneration. It
137 provides clear imaging of regenerated axons from the optical slices scanned by confocal
138 microscope for counting (Supplementary Movie 1). It also allowed us to determine the longest 5
139 axons or single axon growth along the nerve from z-stack of the whole nerve (Figure 1).
140
141 **Quantitation of axon regeneration:** Cleared optic nerves were examined on a confocal
142 microscope by scanning through individual optical slices. Green pseudo-color was used for
143 CTB-labeled axons in all the optic nerve images of this study for clear visual observation.
144 Stacked images were taken at 5 μm increments, a total of 50-100 optical slices for each optic
145 nerve. The number of CTB labeled axons at 0.5 mm from the crush site were counted in at least
146 6 sections per case and calculated by the equation ($\Sigma ad = \pi r^2 * [\text{average axons/mm}]/t$) as
147 described by Leon et al. in 2000 [31]. 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*, was estimated by summing over all sections. Since we used confocal images instead of longitudinal cross sections described in previous studies[3, 4], the optical resolution in *z* (0.5µm) was considered as the *t* (thickness of the slide) in the equation. In some strains very few axons were observed 1mm from the crush site, and for this reason we used direct counts of axons as a measure of regeneration. We also measured the distance that 5 axons had grown down the nerve and the longest distance a single axon reached for each nerve from z-stack image of the whole nerve.

Transfection Efficiency of AAV-shPTEN-GFP: C57BL/6J 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) 1-hour room temperature and placed in primary antibodies RBPMS (Millipore, Cat. # ABN1376) at 1:1000 and GFP (Novus Biologicals, CAT #NB100-1770) at 1:1000 4°C overnight. The retinas were rinsed with PBS and placed in secondary antibodies, (Anti-Goat IgG(H+L) CFTM 488A, Sigma, Cat. #SAB4600032 and Alexa Fluor 594 AffiniPure Donkey Anti-Guinea, Jackson Immunoresearch, Cat. #706-585-1148) at 1:1000 for 1 hour at room temperature. After three washes of 15 minutes each, retinas were flat mounted and cover-slipped using Fuoromount - G (Southern Biotech, Cat. #0100-01) as a mounting medium. Four confocal images were taken in each quadrant at 2 mm away from the optic nerve of each retina. Four retinas from 4 mice of each strain were included. Cell number 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 ($GFP^{+}RBPMS^{+}$ cells) divided by total number of RGCs ($RBPMS^{+}$ cells).

178 **Bioinformatic analysis of known regeneration genes in the BXD strain set:** By searching
179 the literature, we generated a list of genes that are known to have effects on optic nerve
180 regeneration either directly or indirectly (Table 1). All those genes were examined for high
181 Likelihood Ratio Statistics (LRS) scores and cis Quantitative Trait Loci (cis-QTLs) using the
182 GeneNetwork database. They were then put into the SNP (Single Nucleotide Polymorphism)
183 browser of GeneNetwork as well as UCSC genome browser (Mouse, GRCm38/mm10) to
184 identify non-synonymous SNPs between C57BL/6J and DBA/2J. All the identified rsIDs of
185 non-synonymous SNPs were then put into Ensembl (www.ensembl.org/) for SIFT analysis [34]
186 to predict whether the SNP affects protein function.
187
188 **Statistical Analysis:** Data are presented as Mean ± SE (Standard Error of the Mean).
189 Differences in axon counts and regeneration distance were analyzed by Mann-Whitney U test.
190 The differences in transfection efficiency were analyzed by 2- independent sample t-test using
191 SPSS (Statistical Package for the Social Sciences) statistical package 24.0 (SPSS, IBM,
192 Chicago, IL, USA). A value of $p<0.05$ was considered statistically significant.
193

RESULTS

Genetic Background Modulates RGC Axon Regeneration: 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 a genetic reference panel due to distinct advantages these strains have to offer. The first advantage is there are over 80 well characterized strains of mice available through Jackson laboratories. The current set of strains is far larger than any other mature RI resource and will now allow for sub-megabase mapping resolution. The second benefit of the BXD strains are the large microarray datasets specifically for the eye (HEIMED database[14]; HEI Retina Database [35]; Optic Nerve Crush Database[23]; and the DoD Normal Retina Database[36]) along with the numerous ocular phenotypes RGC numbers, 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 mice) 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 both the C57BL/6J mouse and the DBA/2J mouse (Figure 2). For all mice, the 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 significant regenerative response, both in the number of axons counted at 0.5mm and 1mm (Figure 4), as well in the distance the axons traveled (Figure 5). These data demonstrate that the regeneration treatment, knocking down *Pten* and inducing a mild inflammation by injecting of zymosan and CPT-cAMP, produces significantly more regenerating axons than observed in control animals that did not receive treatment.

The number of axons at 0.5mm and 1mm distal to the crush, 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 4, there is a considerable difference between strains on the total number of axons reaching 0.5mm and 1.0mm. The strain with the least number of axons in both cases was BXD

225 102. At both distances from the crush site, the strain with the greatest number of axons was
226 BXD29. The difference was significant (*P*=0.014 Mann-Whitney U test) in the number of
227 regenerated axons reaching 0.5 mm (from a low of 1487.6 ± 264.9 axons in BXD102 to a high of
228 4175.8 ± 648.6 axons in BXD29) from the crush site. There was also a significant difference
229 (*P*=0.007 Mann-Whitney U test) in the number of axons at 1mm, from a low of 1±0 axons in
230 BXD102 to a high of 12.6 ± 0.6 axons in BXD29. The two strains that displayed the least and
231 most robust (BXD102 and BXD29) regenerative response are illustrated in Figure 3.
232
233 The total length of a regenerating axon was also measured. This measure may provide an
234 estimate of the rate at which the axon can grow down the injured optic nerve. When we
235 examined axon length there was also a clear difference in growth across the BXD strains (Figure
236 2, Figure 5). In the control animals, virtually no regenerating axons were observed. When we
237 examined the distance a minimum of 5 axons traveled, a significant difference was observed
238 across the BXD strains. The strain with the shortest regenerating 5 axons was BXD102 with a
239 mean distance of 787.2 ± 46.5µm, and the strain with the longest group of 5 axons was BXD29
240 with a mean distance of 2025.5 ± 223.3µm (*P*=0.014 Mann-Whitney U test). A similar result was
241 observed when examining the distance of the longest single axon traveled in the nerve, with
242 BXD102 having the shortest average distance (1107 ± 40.6µm) and BXD29 the longest (2386.8
243 ± 162.6µm, *P*=0.014 Mann-Whitney U test). Thus, the ability of axonal regeneration (both the
244 number of regenerating axons and the distance traveled) is affected by the genetic backgrounds
245 in the BXD strains with BXD102 having the least regeneration and BXD29 having the most
246 robust regenerative response. These data revealed that genetic background can have a striking
247 effect on the regenerative capacity of axons within the optic nerve.
248
249 **Transfection Efficiency of AAV-shPTEN-GFP:** One possible explanation for the difference
250 is axonal regeneration is a differential transfection of the retinas from strain to strain by the
251 AAV-shPTEN-GFP vector. To control for this possibility, we examined the transfection
252 efficiency and level of *Pten* knock down in the strain with the most robust axon regeneration
253 (BXD29) and another strain with moderate axon regeneration (C57BL/6J). There was no
254 statistically significant difference between the two strains. For the BXD29 strain (n=4) the
255 mean transfection rates was 51.6%±1.3% and for the C57BL/6J strain (n=4) the mean

transfection rate was 53.3% ± 1.7% (Figure 6), indicating that the difference of regeneration response is not due to different transfection efficiency.

Potential Contribution of Known Genes Affecting Axon Regeneration BXD Strains: Previous studies have identified a number of 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 having non-synonymous 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. We usually use the LRS (Likelihood Ratio Statistic) score to represent the association or linkage between differences in traits and differences in particular genotype markers or specific genes. While a statistically significant cutoff can only be determined through permutation tests, LRS scores of >17 usually approximate the significance threshold of $p<0.05$ 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 of the expression level of this gene will have a higher chance to alter the phenotype, which in our 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 non-synonymous SNPs. A non-synonymous 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 non-synonymous

287 SNPs between C57BL/6J and DBA/2J. The SIFT analysis[34] revealed that only two of the
288 eight genes, *Mapk10* (JNK3) and *Rtn4* (NOGO), had SNPs that was predicted to likely affect
289 protein structure/function (rs36844177 in *Mapk10,* SIFT=0.01 and rs29465940 in *Rtn4*,
290 SIFT=0.03). Thus, in the BXD strain set, only three genes known to be associated with axonal
291 regeneration, *Fgf2, Mapk10* (JNK3), and *Rtn4* (NOGO), are actively different between
292 C57BL/6J and DBA/2J mice and potentially contribute to the different response of axonal
293 regeneration across the BXD strains.
294
295
296 **DISCUSSION**
297
298 Over the past several years, advances in optic nerve axon regeneration have taken what was
299 once thought of as an unachievable goal to the point where axonal regrowth after injury is a
300 reality. Several different protocols are being used to promote axonal regeneration[2, 5, 37]. In
301 the present study, we chose a popular protocol developed by others[3, 4] that involves
302 knocking down *Pten* and causing a mild inflammatory response. The BXD recombinant strains
303 are ideal for testing the effects of genetic background with the protocol of knocking down *Pten*,
304 for there are not significant differences in *Pten* between the C57BL/6J strain and the DBA/2J
305 strains. There are no non-synonymous SNPs found in the *Pten* gene between C57BL/6J and
306 DBA/2J mice. Furthermore, there is a similar level of expression of *Pten* mRNA across all of
307 the BXD strains in the DoD normal retina datasets housed on GeneNetwork. We also
308 examined transfection efficiency in two strains (BXD29 and C57BL/6J) that respond
309 differently to the regeneration treatment. There was no difference in transfection efficiency
310 between these two strains. Thus, the difference in the axon regeneration we observed between
311 the different BXD strains cannot be explained by the expression levels of *Pten* in the strains or
312 a differential level of transfection by the AAV2 vector. This leaves only one possibility that
313 the difference in axonal regeneration we observed is due to the specific segregation of
314 genomic elements cross the BXD strains.
315
316 Using the BXD strains we were able to demonstrate the effect of genetic background on the
317 regenerative capacity of axons in the optic nerve. In all strains tested, the amount of regeneration

318 was considerably greater than that observed in mice that did not receive the
319 *Pten*/Zymosan/cAMP treatment. The regeneration responses of C57BL/6J mice we observed
320 were not as strong as described in other studies, a possible reason could be that we were using
321 AAV-shPTEN-GFP to knock down *Pten* instead of cre recombinase-mediated *Pten* knock out.
322 The other factor to be noticed is that the age of mice we used are over 60 days at the time of
323 initial treatment, much older than reported in other studies[3, 4]. This also provides strong
324 evidence that the regeneration response can happen not only in young adult mice but also in
325 older mice. Among the strains treated to promote regeneration, some strains, like BXD102,
326 showed a modest regenerative response; while other strains, like BXD 29, consistently
327 demonstrated a high number of regenerating axons and axons that traveled longer distances
328 down the injured optic nerve. When the response of the parental strains C57BL/6J and DBA/2J
329 was compared to the BXD strains with extreme regenerative responses, there was a clear
330 indication of genetic transgression. If we look at the number of axons at 0.5mm and 1mm from
331 the crush site (Figure 4), there are BXD strains that have fewer axons and BXD strains that have
332 more axons than the parental strains. This difference in regeneration is indicative of genetic
333 transgression. These data reveal that it is not a single genomic locus causing the variability in the
334 regenerative response, for if that were the case the extremes would be similar to the parental
335 strains. This is a clear indication that multiple genomic loci are segregating across the BXD
336 strains to affect the regenerative response of the optic nerve axons. Thus, axon regeneration is a
337 complex trait with multiple modulating genomic loci in the BXD strains.
338
339 A complex trait could be driven by a handful of protein coding genes as well as noncoding
340 variants that presumably affect gene regulation[38]. Multiple genes have been identified in
341 recent years to have an impact on axon regeneration (Table 1). It is possible that some or all of
342 these pathways are varying in the BXD strains and are influencing the outcome of the induced
343 regeneration observed in the present study. With the DoD normal retina datasets[36] and the
344 bioinformatic tools hosted on GeneNetwork, we examined all of the known regeneration-related
345 genes to determine if they were able to modulate the regenerative response across the strains by
346 having either cis-QTLs (differentially expressed genes) or non-synonymous SNPs that would
347 affect protein function. Of all of the known genes known to alter the regenerative response of
348 optic nerve axons, only two were potential candidates for modulating regeneration in the BXD

349 strains. The only cis-QTL in the list of genes was *Fgf2* (LRS = 67.8). SNP analysis identified 8
350 genes with nonsynonymous SNPs in the list of regeneration genes (Table 1). All of the
351 nonsynonymous SNPs were examined using a SIFT analysis and only 2 SNPs (rs36844177 in
352 *Mapk10*(JNK3) and rs29465940 in *Rtn4*(NOGO)) was predicted to alter protein function.
353 Therefore, three possible genomic elements that could be affecting regeneration in the BXD
354 strains are *Fgf2* (Chr3, 37.3 Mb), *Mapk10* (Chr5, 102.9Mb) and *Rtn4* (Chr11, 29.7Mb). Beyond
355 all these, we believe that there are still unknown genomic elements modulating the regenerative
356 response of the axons.
357
358 In conclusion, the ability of optic nerve regeneration response to injury is different across BXD
359 strains given different genetic background. Quantitative trait analysis may provide us with new
360 insights to axon regeneration, and maybe new loci of novel genes or non-coding elements that
361 are involved in axon regeneration. The BXD strains are a good model system and a useful
362 genetic reference panel to study the regeneration of axons in the optic nerve.
363
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368
369 **Conflicts of Interest:** The authors declare that they have no competing interests.
370

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607

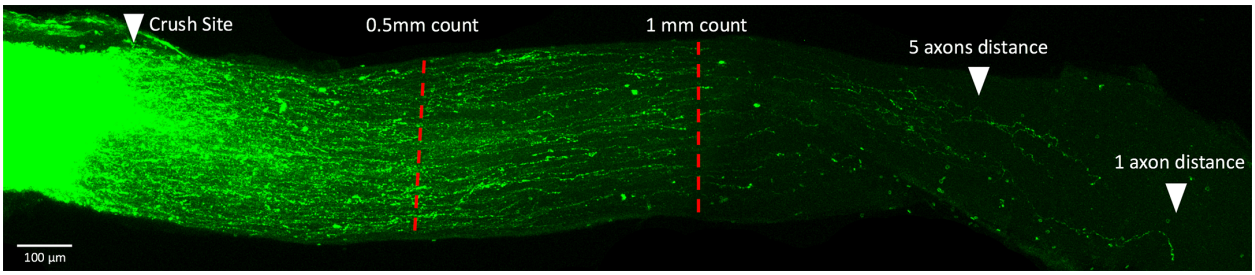
608

609 Table 1. Genetic characteristics of genes that are known to affect optic nerve regeneration
610 between C57BL/6J and DBA/2J mice.

Gene with cis-QTL	*Fgf2*[39]
Genes with Non-synonymous SNPs	*Mapk10* [40], *Rtn4*[41, 42], *Ctgf*[11], *Tlr2*[43], *Rock1*[44], *Rock2*[44, 45], *Clec7a*[43], *Csf2*[46]
Other investigated genes (Genes that are found to have neither cis-QTL nor Non-synonymous SNPs)	*Braf[47, 48], Bcl2[9], Myc[49], Cntf[50] ,Dpysl2[51], Ecel1[52], Map3k12[53], Egfr [17], Gsk3b[54, 55], Hhex [56], Hnrnpk [57], Spp1[58], Stat3[2, 59, 60], Klf4[2, 60, 61], Klf6[2, 61, 62], Klf7[2, 61, 62], Klf9[2, 61], Lif[50, 63], Rtn4r[10, 64, 65], Ntn1[66, 67], Pten[1], Ptprs[68], Rhoa[69-71], Cxcl12[72], Set[73], Socs3[37, 74], Sox11[5, 75], Tet1[76], Tet3[76], Wnt10b[77], Slc30a3[78], Bag1 [79]*

611

612 **Figures:**



613

614 **Figure 1.** The regenerating axons in the optic nerve 14 days after optic nerve crush. We have
615 indicated the regions of the nerve where axons were counted as well as the distance that 5 axons
616 or 1 axon regenerated down the nerve. Scale bar represents 100 µm.

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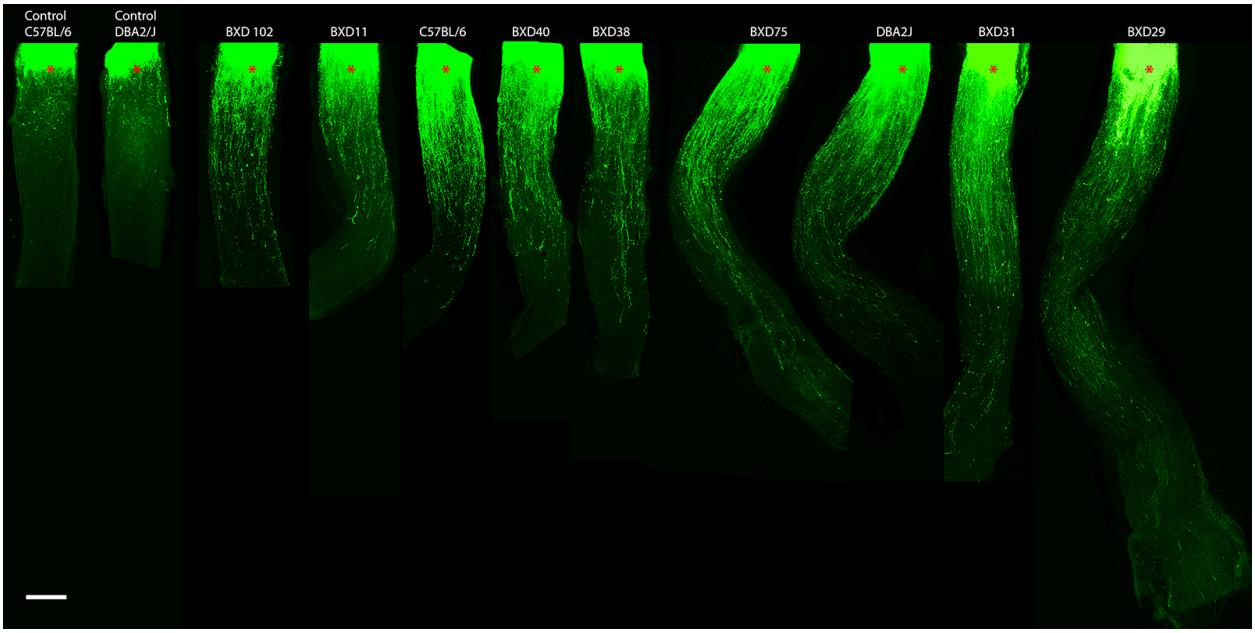


618

619 **Figure 2**. Genetic background affects regenerating axons in the optic nerve following crush.
620 The figure is a series of photomicrographs from 11 optic nerves selected from 9 different
621 strains of mice. The first two images on the far left are from control mice that did not receive
622 the regeneration treatment prior to optic nerve crush (Control C57BL/6J and Control DBA/2J).
623 All of the remaining nerves were from animals in which *Pten* was knocked-down and a mild
624 inflammatory response was induced. The strain with the least regeneration was BXD102 and
625 the strain with the greatest regeneration was BXD29. Red asterisks represent the crush site.
626 The scale bar represents 200µm.

627

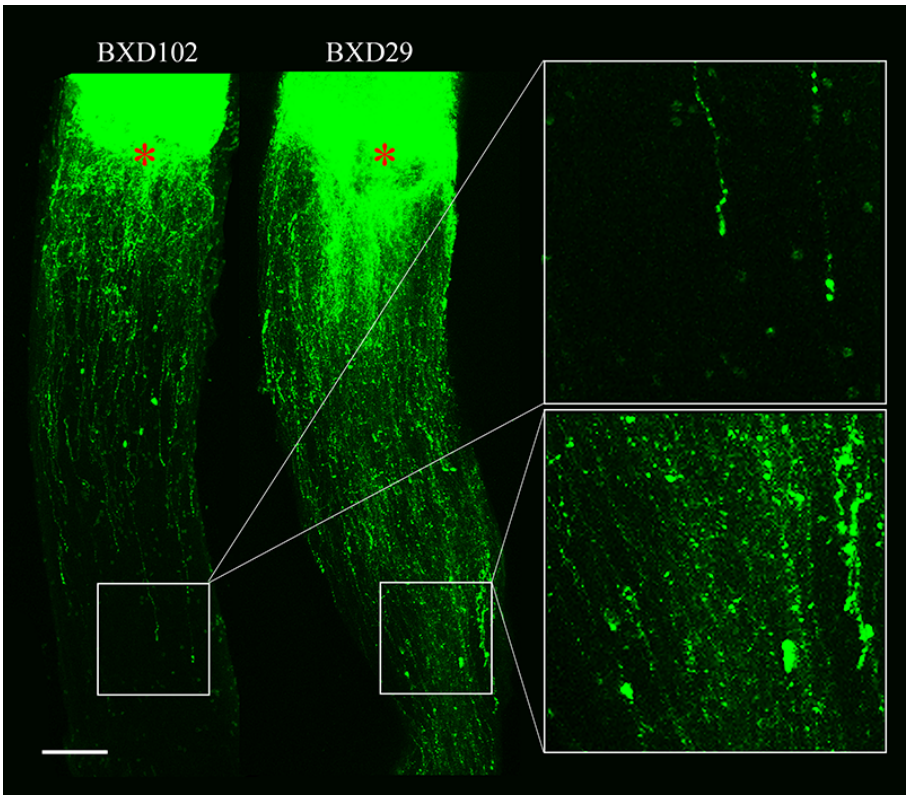


Figure 3. Comparison of regenerated axons in strains with the least regeneration (BXD102) and the greatest regeneration (BXD29). Higher magnification of axons at 1mm (Boxed region) from crush site were shown relatively. The scale bar represents 100µm.

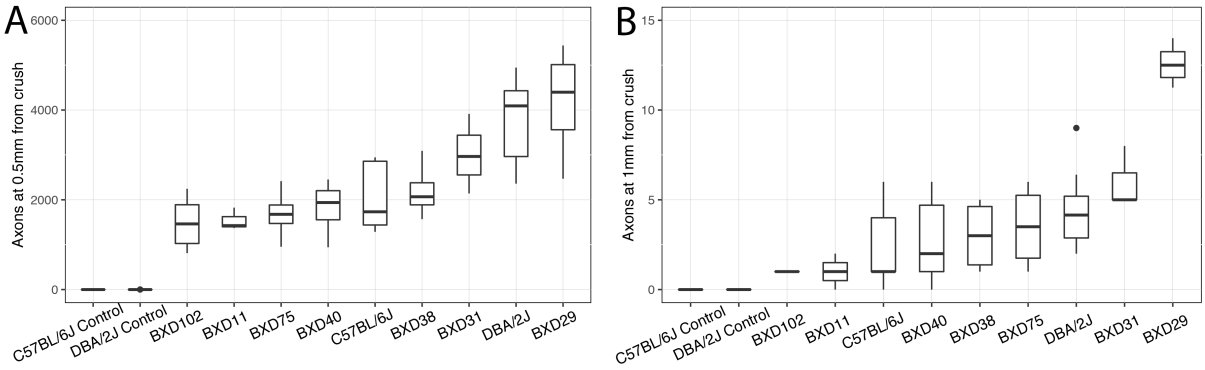


Figure 4: The number of axons at 0.5mm (A) and 1mm (B) from the crush site in two control strains (DBA/2J and C57BL/6J untreated mice) and in 9 strains treated with the regeneration protocol. Boxplots show median, 25th and 75th percentile, maximum, and minimum values for each BXD RI strain.

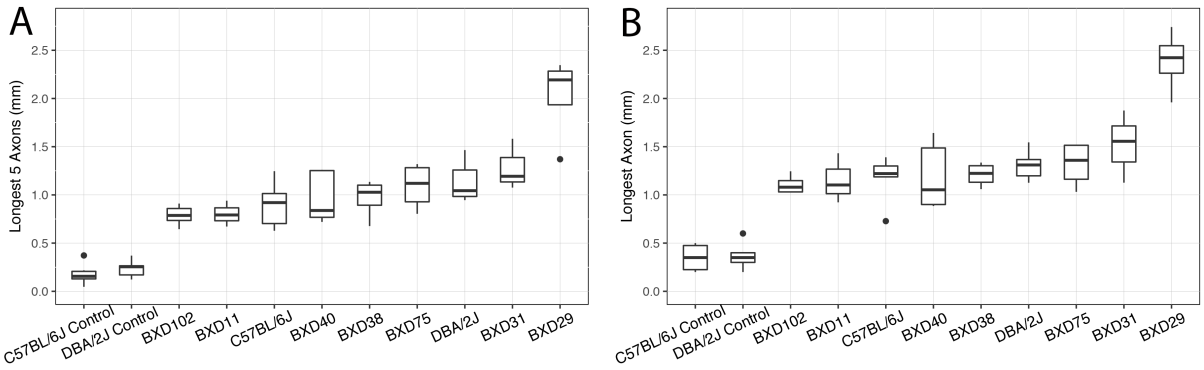


Figure 5: The longest distance that 5 axons regenerated (A) and the longest regeneration for a single axon (B) are shown for the two control strains (DBA/2J and C57BL/6J untreated mice) and in 9 strains treated with the regeneration protocol. Boxplots show median, 25th and 75th percentile, maximum, and minimum values for each BXD RI strain.

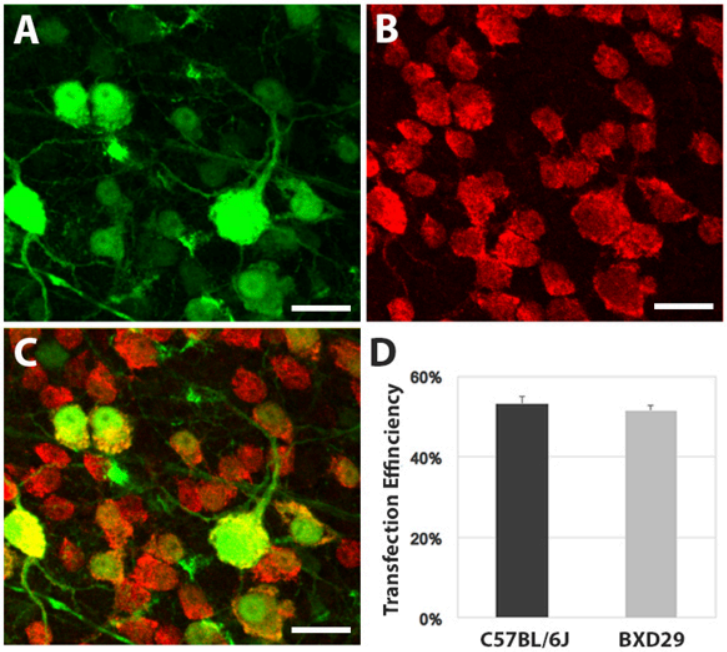
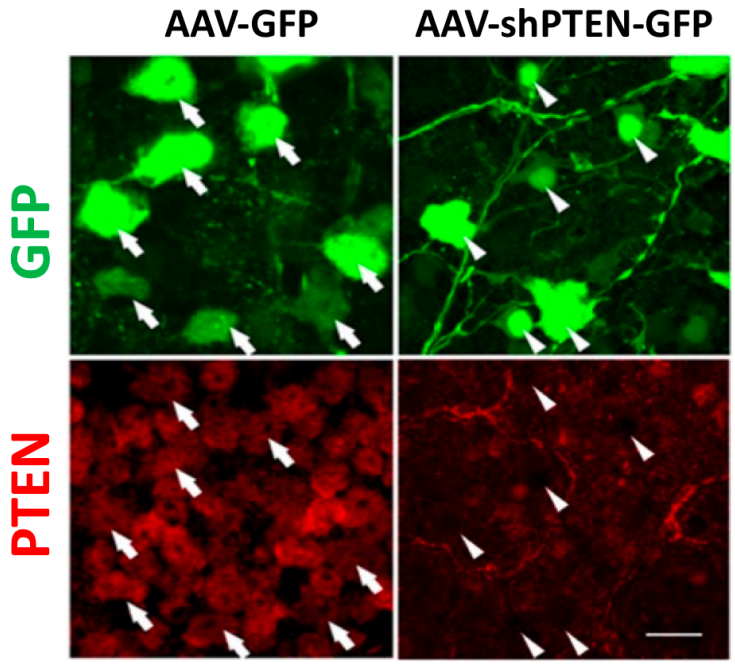


Figure 6. Transfection Efficiency of AAV-shPTEN-GFP. AAV transfected cells are labeled by GFP in green (A) and total number of RGCs are labeled by RBPMS in red (B). Merged channel shown in (C). No statistical difference of transfection efficiency was found between C57BL/6J mice and BXD29 mice (D). Scale bar represents 100 µm.

651 **Supplementary data:**
652
653 **Supplementary Method:**
654 **Evaluation of *Pten* knock down by immunofluorescence staining**
655 Six C57BL/6J mice were deeply anesthetized with a mixture of 15 mg/kg of xylazine and 100
656 mg/kg of ketamine and perfused through the heart with saline followed by 4% paraformaldehyde
657 in phosphate buffer (pH 7.3). For the retinal flat mounts, the retinas were removed from the
658 globe and rinsed in PBS with 1% Triton X-100, blocked in 5% Bovine Serum Albumin (BSA) 1
659 hour room temperature and placed in primary antibodies *Pten* (Cell Signaling, Cat. # 9559) at
660 1:200 and GFP (Novus Biologicals, CAT #NB100-1770) at 1:1000 4°C overnight. The retinas
661 were rinsed with PBS and placed in secondary antibodies, (Anti-Goat IgG(H+L) CFTM 488A,
662 Sigma, Cat. #SAB4600032 and Alexa Fluor 594 AffiniPure Donkey Anti-Rabbit, Jackson
663 Immunoresearch, Cat. #711-585-152) at 1:1000 and To-PRO-3 Iodide (Molecular Probes, Cat. #
664 T3605) was applied 1:1000 as a nuclear counterstain for 1 hour at room temperature. After three
665 washes of 15 minutes each, retinas were flat mounted and cover-slipped using Fuoromount - G
666 (Southern Biotech, Cat. #0100-01) as a mounting medium.
667



668
669 **Supplemental figure 1.** To determine if *Pten* was knocked down by our AAV treatment, we stained the retina for
670 *Pten* (Red) in retinas injected with the AAV-GFP vector (control) or AAV-shPTEN-GFP to knock down *Pten*. In
671 the control retina. all of the GFP positive cells were also well labeled for *Pten*. In the retinas that received the
672 AAV-shPTEN-GFP, the transfected cells (GFP positive) had low levels of *Pten* staining indicating that the vector
673 had in fact decreased the expression of *Pten*.
674
675
676

677 **Supplementary Table 1. Summary of optic nerve regeneration in the BXD strains**

Strains	N	Age (Days)*	Axons at 0.5mm from crush	Axons at 1mm from crush	Longest 5 Axons (µm)	Longest 1 Axon (µm)
C57BL/6J Control	6	67	0±0	0±0	179.2±45	350±56.3
DBA/2J Control	6	63	0±0	0±0	235.3±36.8	366.7±55.8
BXD102	5	61	1487.6±264.9	1±0	787.2±46.5	1107±40.6
BXD11	5	60	1540.7±143	1±0.6	801.3±77.5	1152.7±149
C57BL/6J	5	67	2052.4±354.5	2.4±1.1	902.2±110.9	1165.2±114.8
BXD40	9	62	1818.4±208.5	2.7±0.8	962.1±87.5	1184.8±116.6
BXD38	4	67	2199.3±320.7	3±1	966.8±103.1	1210.5±63.2
BXD75	5	65	1680.8±298.7	3.5±1.2	1090.8±122.8	1318±120.3
DBA/2J	8	63	3777.8±352.7	4.5±0.8	1125.5±66.4	1298.1±49.2
BXD31	4	61	3007±511.7	6±1	1283.7±152.9	1519.3±216.7
BXD29	4	65	4175.8±648.6	12.6±0.6	2025.5±223.3	2386.8±162.6

678 *: Ages of mice at the initial regeneration treatment (Day 0)

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680 **Supplementary Movie 1**

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