

**Supplemental Information**

**Codon-Optimized RPGR Improves Stability  
and Efficacy of AAV8 Gene Therapy in Two  
Mouse Models of X-Linked Retinitis Pigmentosa**

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## **Supplemental Materials**

Fig. S1. Overview over wild-type *RPGR* at Xp11.4 and the changes to optimise the coding sequence of *RPGR*<sup>ORF15</sup>.

Fig. S2. Sequencing of the *wtRPGR*<sup>ORF15</sup> cloning vector.

Fig. S3. Superior sequence fidelity of *coRPGR*<sup>ORF15</sup> over *wtRPGR*<sup>ORF15</sup>.

Fig. S4. Independent confirmation of superior sequence stability of *coRPGR*<sup>ORF15</sup>.

Fig. S5. Flow cytometric analysis of RPGR<sup>ORF15</sup> expression.

Fig. S6. RPGR gene therapy shows no toxicity in electroretinography (ERG) of unilaterally treated wild-type mice.

Fig. S7. RPGR gene therapy shows no toxicity in electroretinography (ERG) of bilaterally treated wild-type mice.

Fig. S8. Representative retinal images of *C57BL/6J* mice at postnatal month 6 (PM6).

Fig. S9. ERG recordings in *Rpgr*<sup>-/-</sup> mice after *coRPGR*<sup>ORF15</sup> gene therapy.

Fig. S10. ERG recordings in *C57BL/6J*<sup>Rd9/Boc</sup> mice after *coRPGR*<sup>ORF15</sup> gene therapy.

## Supplemental Figure Legends:

**S1.** Overview over wild-type *RPGR* at Xp11.4 and the changes to optimise the coding sequence of *RPGR<sup>ORF15</sup>*. **(A)** *RPGR<sup>ORF15</sup>* is the longest *RPGR* isoform (consensus coding sequences CCDS35229.1) and encodes for an 1152 amino acid protein with distinct domains. The more N-terminal RCC1-like domain is common to all known *RPGR* isoforms, while the glycine/glutamic acid rich domain and the carboxy-terminus are unique for *RPGR<sup>ORF15</sup>*. **(B)** Codon optimisation of *RPGR<sup>ORF15</sup>* leads to significant changes in the primary coding sequence. Here, altered GC frequency (%) is indicated along the full coding sequence of *RPGR<sup>ORF15</sup>* with wild-type *RPGR<sup>ORF15</sup>* indicated on the top (black) and codon optimised *RPGR<sup>ORF15</sup>* (*coRPGR<sup>ORF15</sup>*) at the bottom (red) with grey breaks indicating the changes from the wild-type sequence. **(C)** The full sequence is displayed with *coRPGR<sup>ORF15</sup>* on top (Optimized) indicating the silent substitutions indicated in red, while the wild-type *RPGR<sup>ORF15</sup>* sequence is displayed as reference below (Original).

**S2.** Sequencing of the *wtRPGR<sup>ORF15</sup>* containing cloning vector revealed a 12bp deletion in the ORF15 region (c.3052\_3063del), which would lead to an in-frame loss of four aminoacids (Gly-Arg-Gly-Ser).

**S3.** Superior sequence fidelity of *coRPGR<sup>ORF15</sup>* over *wtRPGR<sup>ORF15</sup>*. **(A)** While all *wtRPGR<sup>ORF15</sup>* plasmid preparations featured at least some mutations, none of the *coRPGR<sup>ORF15</sup>* plasmid preparations was found to harbour any deletion, insertion- or point mutation. **(B)** Percentage of nucleotides within each plasmid sequence with at least 99% (first row), 99.9% (second row) or 99.99% (third row) base call accuracy. These levels of confidence of individual base calls corresponds to the Phred quality scores Q20, Q30 and Q40 respectively. Numbers are reported as mean ± standard deviation. Mean confidence level of base call accuracy (fourth row) and number of expected errors (bottom row) for each plasmid sequence. Numbers are reported as mean ± standard deviation. Statistical analysis was performed with Student t-test (n=4) and corrected for multiple testing using the false discovery rate method by Benjamini et al (1995).

**S4.** Independent confirmation of superior sequence stability of *coRPGR<sup>ORF15</sup>*. The National Genetics Reference Laboratory (NGRL) in Manchester identified multiple potential mutations in the *wtRPGR<sup>ORF15</sup>* construct (top). There were six potential frame shift mutations (two deletions, four insertions) and 74 additional ambiguous base calls. In contrast, the sequence of the *coRPGR<sup>ORF15</sup>* construct was confirmed to be intact with at least two times coverage (bottom). The symbol # indicates use of reverse primers.

**S5.** Flow cytometric analysis of *RPGR<sup>ORF15</sup>* expression. **(A)** HEK293T cells were transfected with either *coRPGR<sup>ORF15</sup>* (*coRPGR*), *wtRPGR<sup>ORF15</sup>* (*wtRPGR*), or *eGFP* containing control plasmids (scale bar = 20µm). **(B)** Harvested cells were immuno-labelled with primary anti-RPGR/secondary fluorescent antibodies and *eGFP* cells were used to set the lower end of the FACS gating for fluorescence in the far-red range as they were incubated with secondary antibody only. Positive controls (naïve HEK293T cells exposed to rabbit anti-βactin and donkey anti-rabbit with conjugated Alexa-Fluor 635) were then used to define the upper end of the fluorescence gate setting. Cells transfected with the *coRPGR<sup>ORF15</sup>* construct (co) showed higher fluorescence intensity than the cells transfected with the wild-type construct (wt). The Shapiro-Wilk test rejected the null-hypothesis for normality of the data sets ( $p < 0.05$ ) and the Kruskal Wallis non-parametric test demonstrated a robust statistical difference between the cohorts ( $p < 0.01$ ,  $n = 9$ ). Box plot (median, box delineates lower and upper quartile, whiskers minimum and maximum) of median fluorescence intensities in arbitrary units [AU].

**S6.** RPGR gene therapy shows no toxicity in electroretinography (ERG) of unilaterally treated wild-type mice. ERG recordings in *C57BL/6J* mice after unilateral subretinal injection of AAV.RK.coRPGR (red) vs. no treatment (black). **(A)** shows data at two months of age (PM2), **(B)** at PM4 and **(C)** at PM6, the last time point tested. Factorial ANOVA for repeated measures retained the null hypothesis (no difference) in all analyses. Lines indicate mean amplitudes ± 95% confidence interval (whiskers).

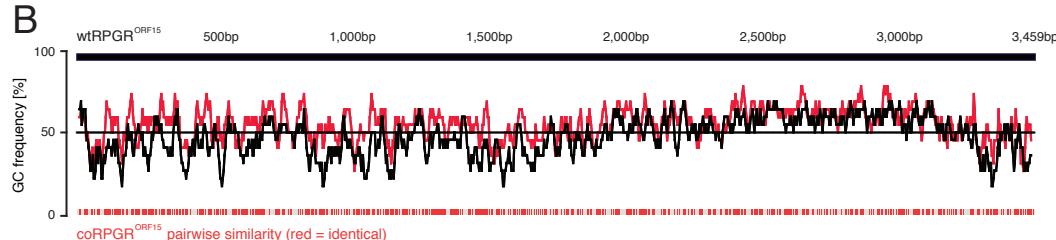
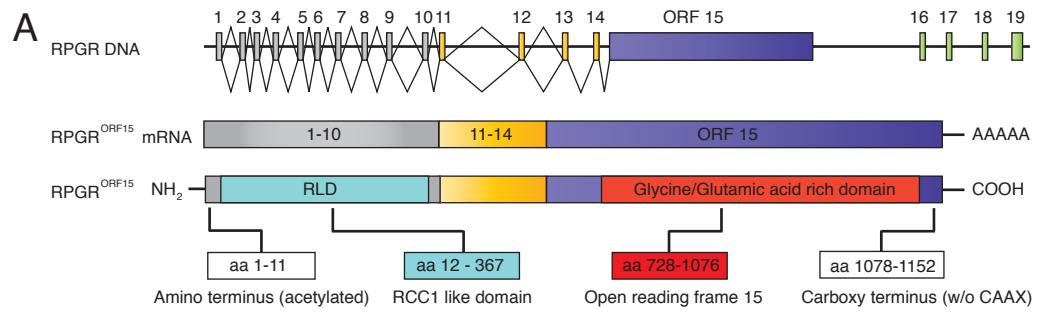
**S7.** RPGR gene therapy shows no toxicity in electroretinography (ERG) of bilaterally treated wild-type mice. ERG recordings in *C57BL/6J* mice after bilateral subretinal injection of AAV.RK.coRPGR (red) and AAV.control (black). **(A)** shows data at two months of age (PM2), **(B)** at PM4 and **(C)** at PM6, the last time point tested. Factorial ANOVA for repeated measures retained the null hypothesis (no difference) in all analyses. Lines indicate mean amplitudes ± 95% confidence interval (whiskers).

**S8.** Representative retinal images of *C57BL/6J* mice at postnatal month 6 (PM6). Two columns represent eyes of the treatment group (left) and sham control group (right). Using scanning laser ophthalmoscopy imaging in the infrared mode, the focal plane was set to inner retina (top row) or outer retina (middle row). Bottom row demonstrates the physiologically weak autofluorescence in wild-type mice.

**S9.** ERG recordings in *Rpgr<sup>-y</sup>* mice after *coRPGR<sup>ORF15</sup>* gene therapy. Top (**A-C**) shows data from unilateral trial; bottom (**D-F**) from bilateral trial. Mean amplitudes ( $\pm$  95% confidence interval) are shown in red for treated eyes and black for untreated or sham treated eyes. (**A** and **D**) show data at two months of age (PM2), (**B** and **E**) at PM4 and (**C** and **F**) at PM6, the last time point tested. Treatment with AAV.RK.coRPGR led to significant improvement of dark adapted ERG amplitudes (left panel in **B-C**) in the unilateral treatment trial. The treatment effect in the light adapted b-wave amplitudes (right panel in **C**) only became apparent at PM6. Amplitudes in treated eyes were consistently higher than in the sham treated eyes (**D-F**), but this only reached significance at PM2 (**D**) and PM6 (**F**) for dark adapted responses and PM4 (**E**) for light adapted responses.

**S10.** ERG recordings in *C57BL/6J<sup>Rd9/Boc</sup>* mice after *coRPGR<sup>ORF15</sup>* gene therapy. Top (**A-C**) shows data from unilateral trial; bottom (**D-F**) from bilateral trial. Mean amplitudes ( $\pm$  95% confidence interval) are shown in red for treated eyes and black for untreated or sham treated eyes. (**A** and **D**) show data at two months of age (PM2), (**B** and **E**) at PM4 and (**C** and **F**) at PM6, the last time point tested. Treatment with AAV.RK.coRPGR led to significant improvement of dark adapted ERG amplitudes (left panel in **B-C**) in the unilateral treatment trial. There was not significant treatment effect in the light adapted amplitudes (right panels) or in dark- or light adapted responses in the bilateral trial. However, amplitudes in treated eyes were consistently higher than in the sham treated eyes without reaching significance levels in this phenotypically mild disease model.

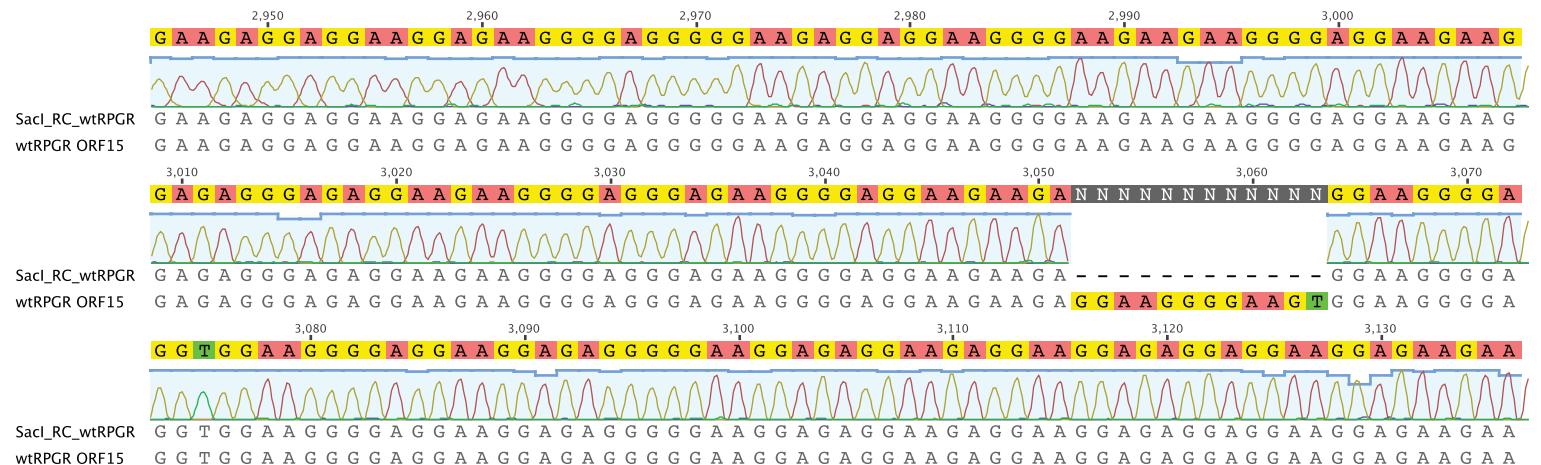
# Figure S1



**C**

	Optimized	Original		Optimized	Original		Optimized	Original	
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61	AAGTTCCGTGAAATAACCCAGGAAATGCTGTAAAACGACGTGCCGTCACCTG	AAATTTCGCTGAAATAATCCCGTAANATTCTGGTTAAAATGATGTCCTGTACATCTT	1801	ATTGAGGAACAGNGGTTGAAAGCJAACGAGGAAAATGTAAGTCACCGAAGGAG	CGGAAGCAGGAGA				
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541	TGCGTCCTTCAGCAGGTGACCATGGAAAGCCAGTCAGTGGATTCTGTGCTACTAT	TGTGTCCTTCAGCAGGTGACCATGGAAACCTGTCCTGGATCTCTGTTGATATTAC	2281	GGCGAGAAGTGAAGCGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAG	GAACGAAAGAAAGGAGA				
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721	ATCCCTGAAAAAGTGATTCAGCTGGCTGGGGAGAGGACATACAGTCGTCCTGACTGAG	ATCCCTGGAGAGGTGATCAAAGTAGCTGTGTTGGAGAGGACATCTGTTCTCACTGGAG	2461	GAGGAAAAGGGCAGCGAGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAG	GGGGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAG				
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961	GATGGGACACGGGAAGCTGGAGCTGGAGCTGGAGGAACTTCACTATCATTCTT	GATGGTGGCACGAAAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG	2701	GAGGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG	GGGGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG				
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1081	ATGGTGGCTTCTGCTGACCTCATGGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGT	ATGGTAGTTTCTGCTCCTCATGTCGTCCTGGTGTGAGGAACTTCACTCTTCT	2821	GGAGAGGAGCGAGGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAG	GGGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAG				
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1201	GTGCTGAGAGAACCTCTGAGTGAAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAG	GTACTGAGAGAACCTCTGAGTGAAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAG	2941	GGGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAG	GGGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAG				
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1441	GAGGCCAAATGCACTGAGAACCTCTGAGTGAAGGAGGAGGAGGAGGAGGAGGAGGAG	GAAGCAGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAG	3181	GAGGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAG	GGGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAG				
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1681	CAGCATGTCAGTCAGGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAG	CAACATGTCACAAAGGGATTTTCATGACGCCAGGAGGAGGAGGAGGAGGAGGAGGAGGAG	3421	TGGACATACTGAGTGAAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAG	GGGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAG				
Original	1681	AAAGCTGAGATGAGTGAAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAG	AAAGCTGAGATGAGTGAAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAG	3421	AAAATGCGCACTGAGTCAAAACGAGCTTTAAACGAGTGTGAAATGAGTGAAGTGAAGTAA	GGGGAG			
Optimized	1681	CAGCATGTCAGTCAGGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAG	AAAGCTGAGATGAGTGAAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAG	3421	TGGACATACTGAGTGAAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAG	GGGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAG			

## Figure S2



## Figure S3

**A**

	<i>wtRPGR</i> <sup>ORF15</sup>	<i>coRPGR</i> <sup>ORF15</sup>
<b>deletions</b> [mean (range)]	1.5 (0 - 4)	<i>nil</i>
<b>insertions</b> [mean (range)]	0.5 (0 - 1)	<i>nil</i>
<b>point mutations</b> [mean (range)]	17.8 (9 - 33)	<i>nil</i>
<b>Total</b> [mean (range)]	19.75 (9 - 38)	<i>nil</i>

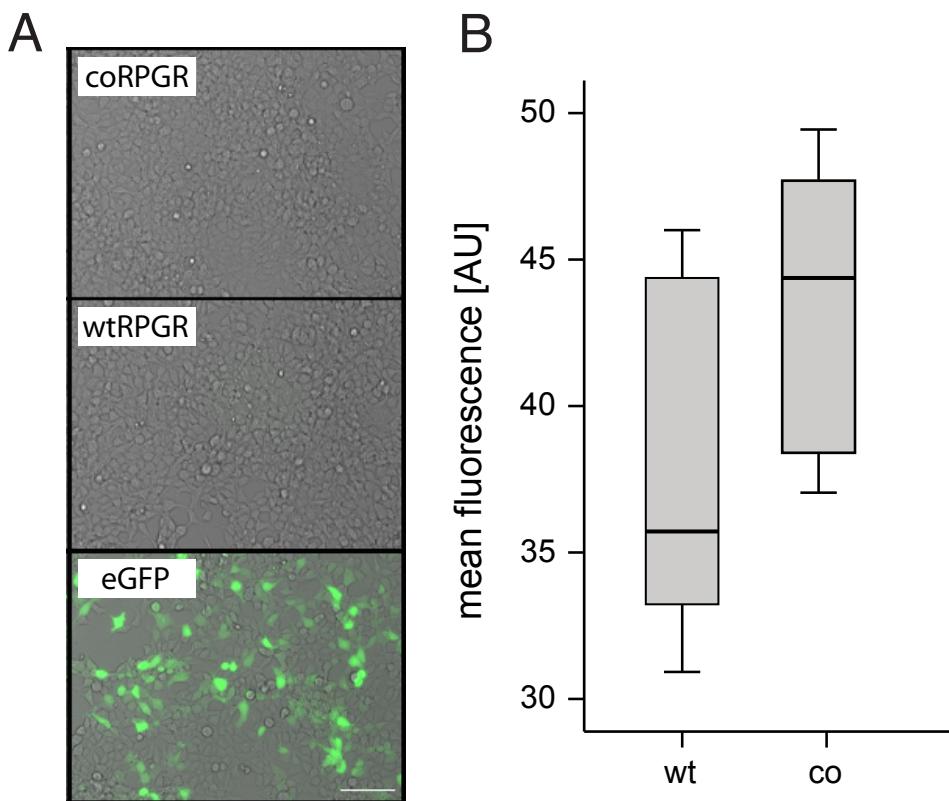
**B**

	<i>wtRPGR</i> <sup>ORF15</sup>	<i>coRPGR</i> <sup>ORF15</sup>	<i>p</i> - value [FDR corrected]
<b>99% (Phred Q20)</b>	$91.3 \pm 1.0$	$96.6 \pm 0.9$	0.0005
<b>99.9% (Phred Q30)</b>	$83.1 \pm 2.6$	$90.5 \pm 2.6$	0.0044
<b>99.99% (Phred Q40)</b>	$73.6 \pm 3.8$	$82.3 \pm 3.0$	0.0054
<b>Confidence Mean</b>	$49.1 \pm 1.2$	$52.4 \pm 1.0$	0.0044
<b>Expected Errors</b>	$82.9 \pm 25.1$	$14.4 \pm 5.1$	0.0023

## Figure S4



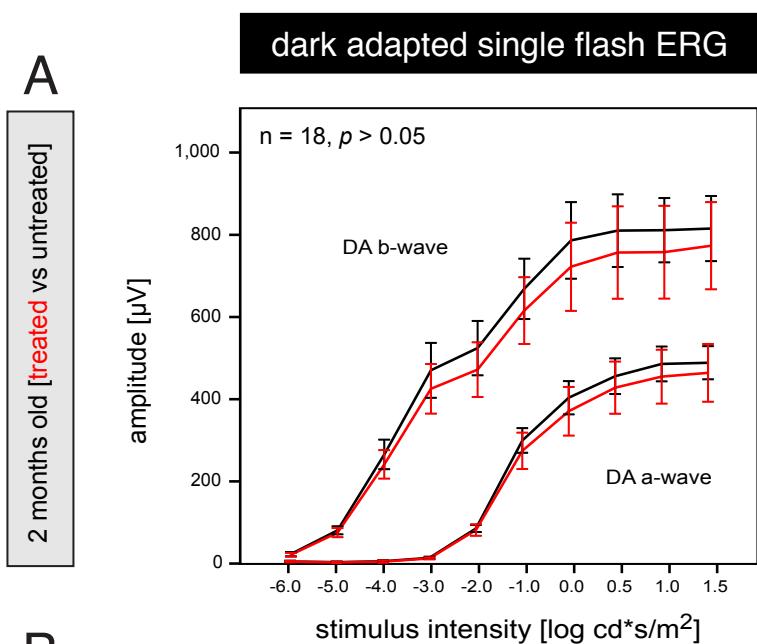
## Figure S5



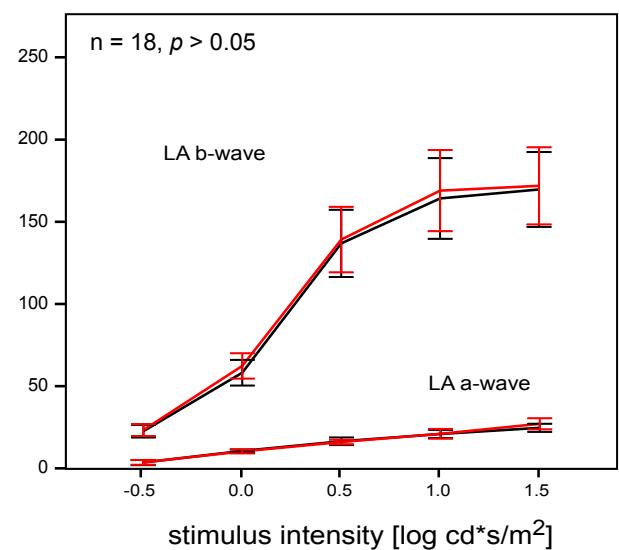
## Figure S6

### C57BL/6J unilateral trial (red = RPGR)

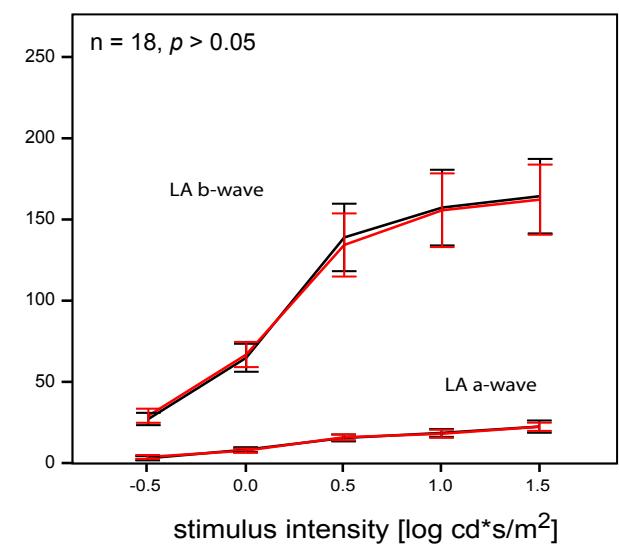
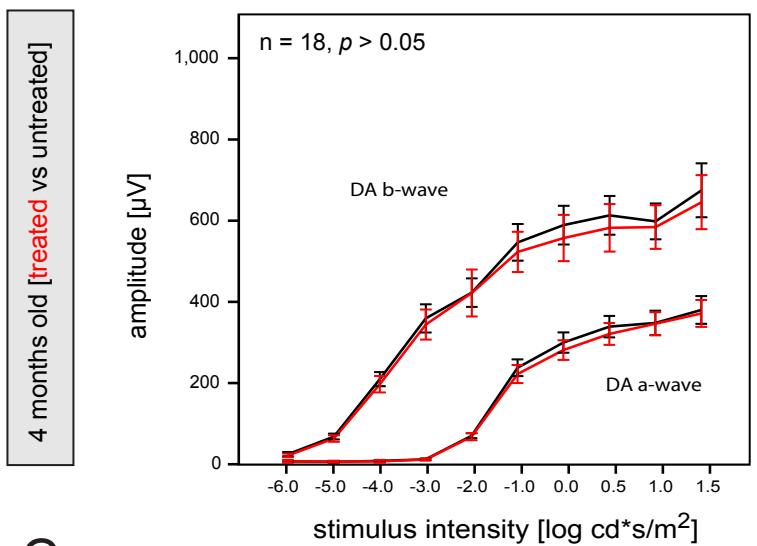
A



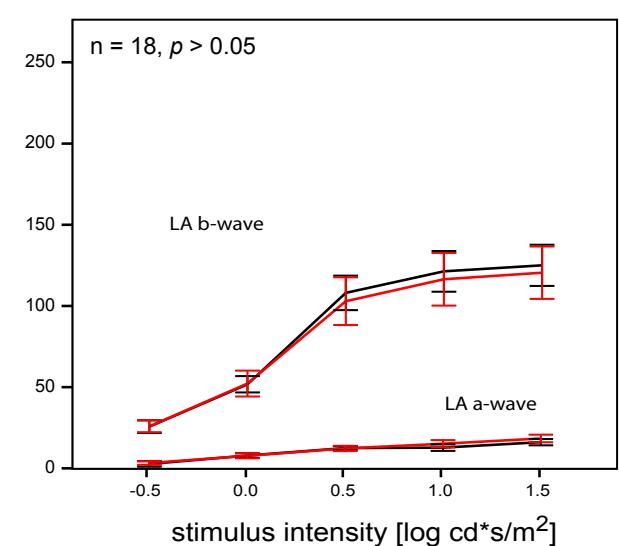
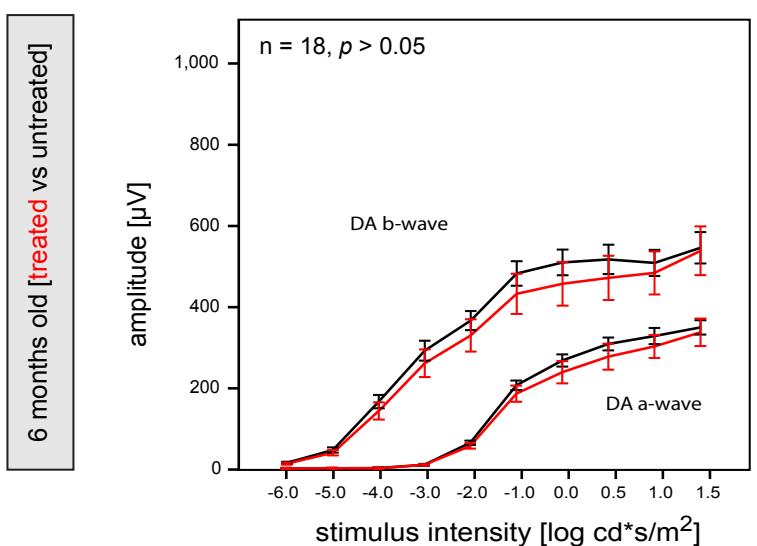
light adapted single flash ERG



B

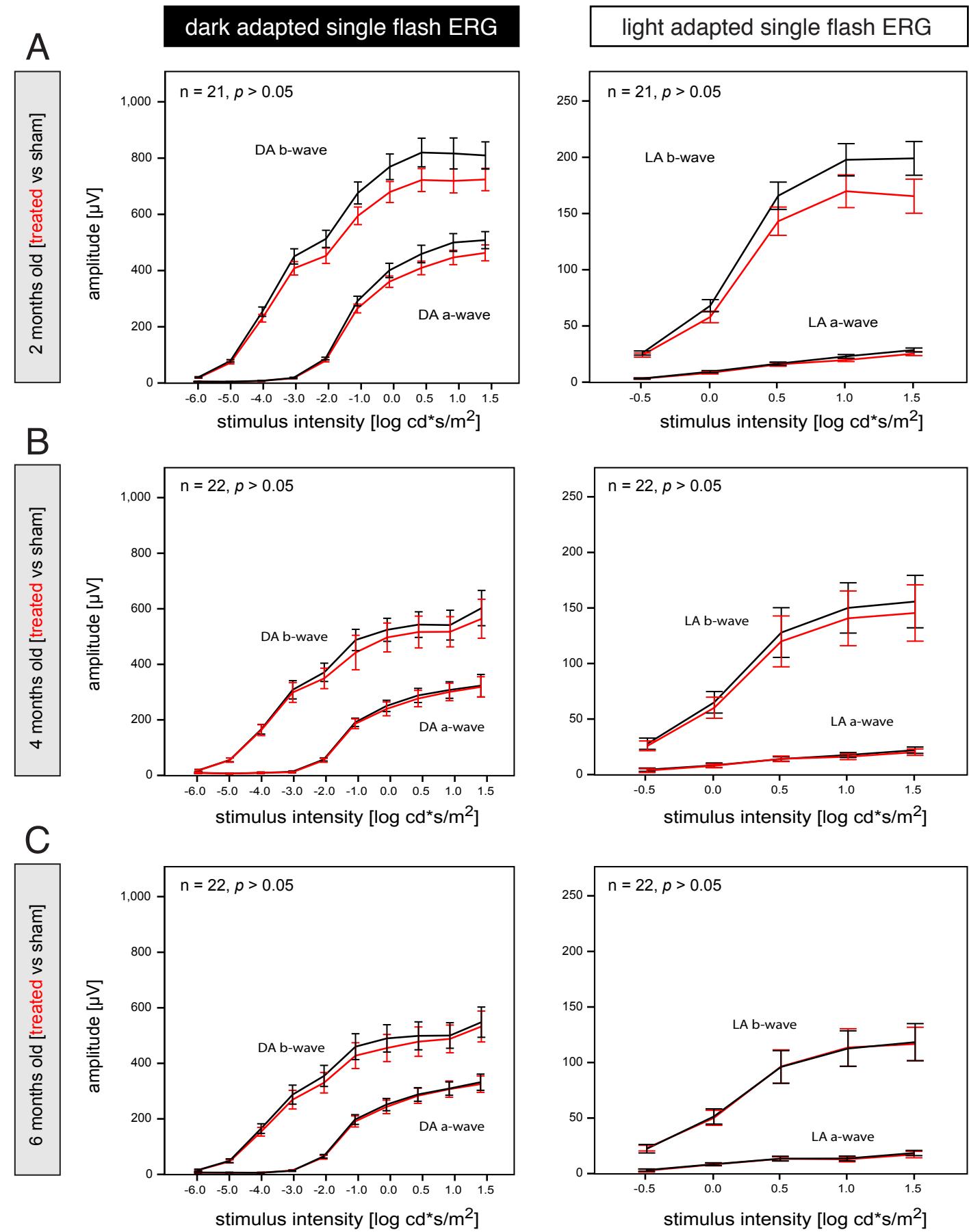


C

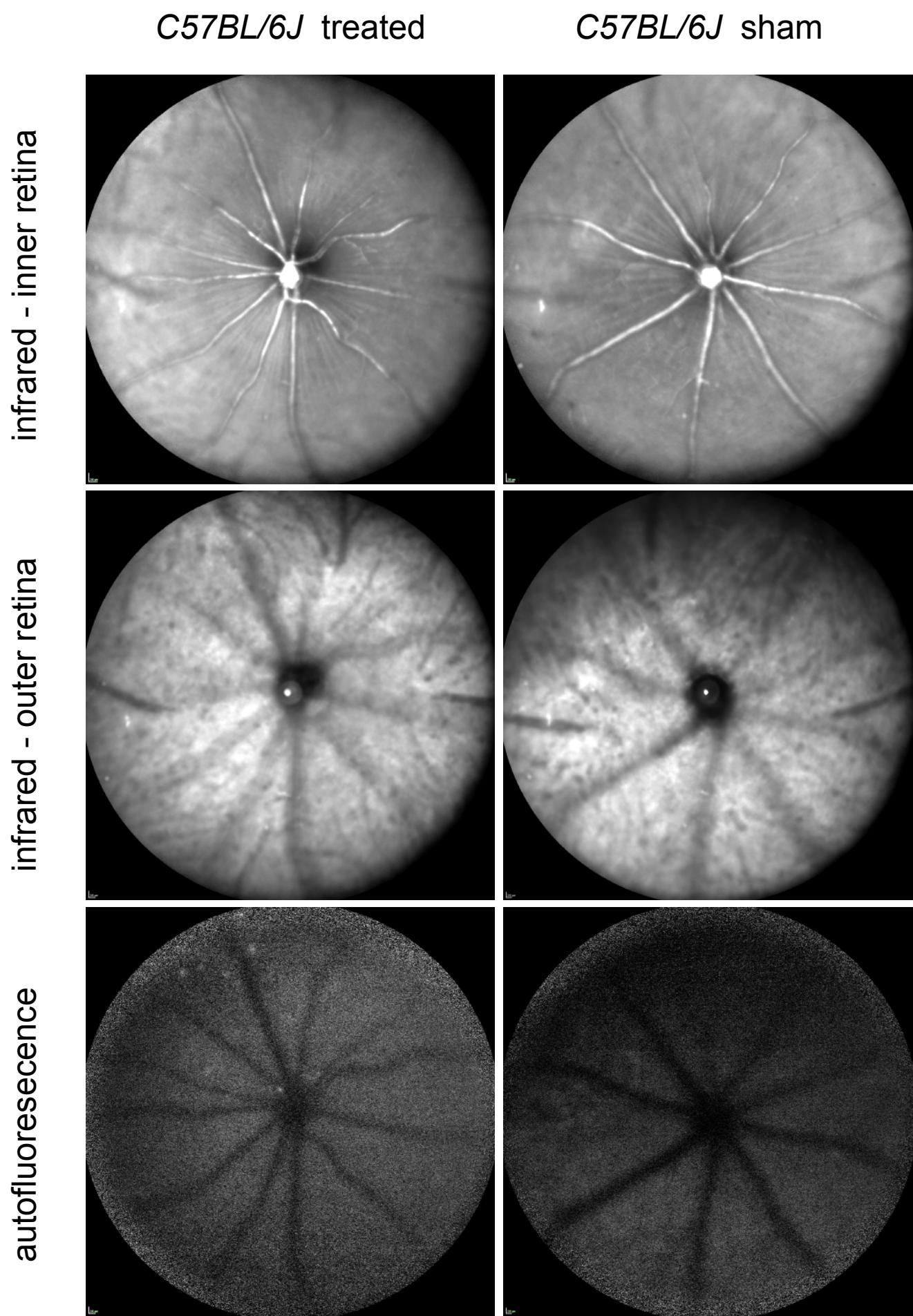


**Figure S7**

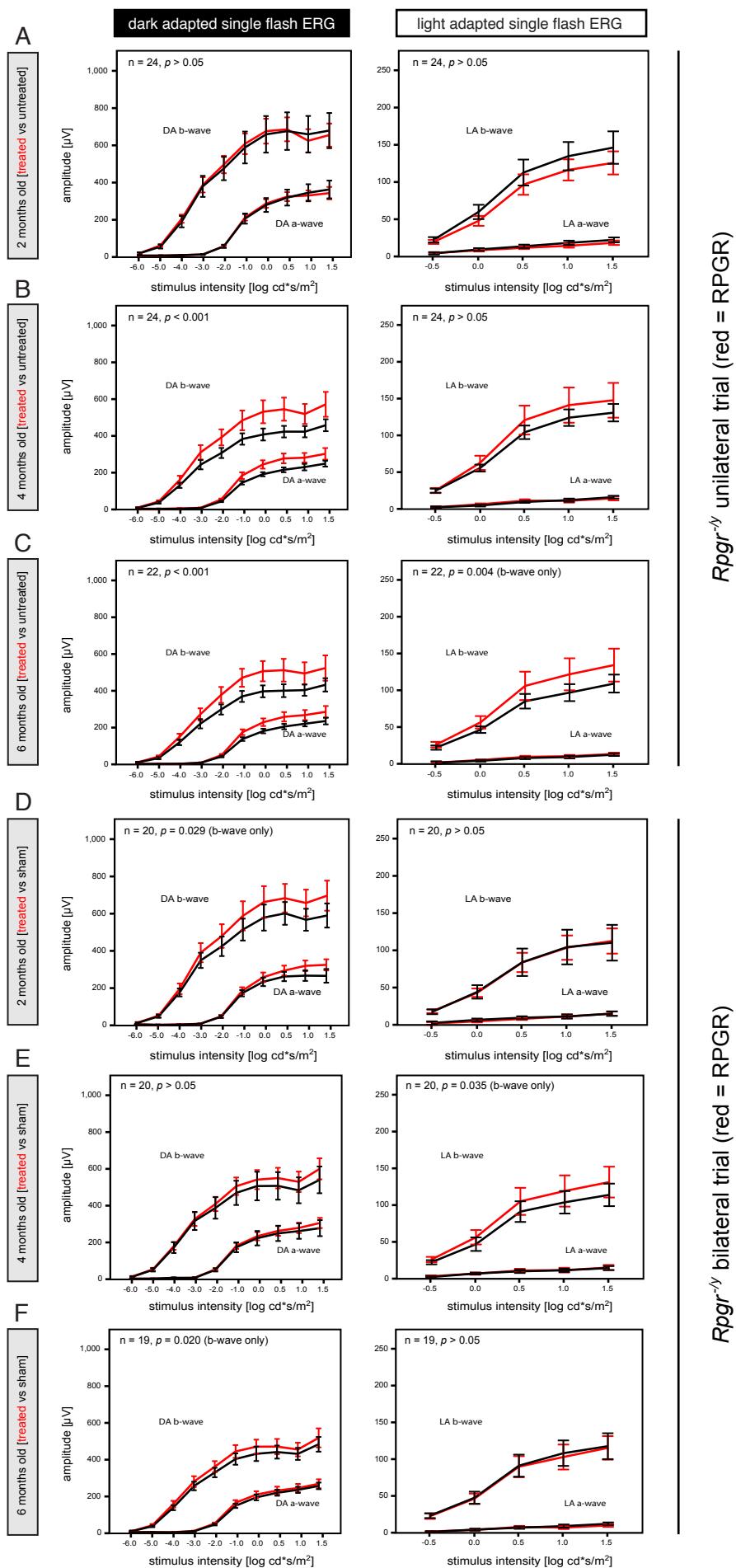
**C57BL/6J bilateral trial (red = RPGR)**



**Figure S8**



# Figure S9



# Figure S10

