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## Accepted Manuscript

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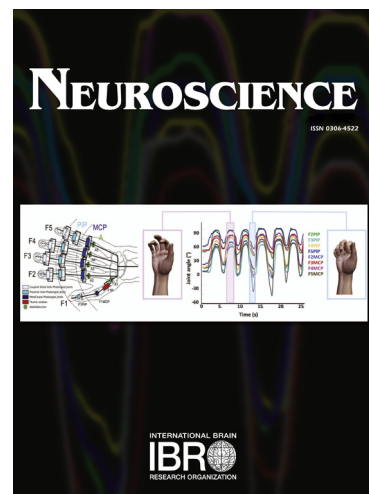
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**Cre recombinase expression or topical tamoxifen treatment do not affect retinal structure and function, neuronal vulnerability or glial reactivity in the mouse eye**

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Key words: Cre recombinase; tamoxifen; retinal neurons; neuronal vulnerability; neuroprotective factors; macro- and microglia cell reactivity

**Abstract**

Mice with a constitutive or tamoxifen-induced Cre recombinase (Cre) expression are frequently used research tools to allow the conditional deletion of target genes via the Cre-loxP system. Here we analyzed for the first time in a comprehensive and comparative way, whether retinal Cre expression or topical tamoxifen treatment itself would cause structural or functional changes, including changes in the expression profiles of molecular markers, glial reactivity and photoreceptor vulnerability. To this end, we characterized the transgenic  $\alpha$ -Cre, *Lmop-Cre* and the tamoxifen inducible *CAGG-CreER<sup>TM</sup>* mouse lines, all having robust Cre expression in the neuronal retina. In addition, we characterized the effects of topical tamoxifen treatment itself in wildtype mice. We performed morphometric analyses, immunohistochemical staining, *in vivo* ERG and angiography analyses and realtime RT-PCR analyses. Furthermore, the influence of Cre recombinase or topical tamoxifen exposure on neuronal vulnerability was studied by using light damage as a model for photoreceptor degeneration. Taken together, neither the expression of Cre, nor topical tamoxifen treatment caused detectable changes in retinal structure and function, the expression profiles of investigated molecular markers, glial reactivity and photoreceptor vulnerability. We conclude that the Cre-loxP system and its induction through tamoxifen is a safe and reliable method to delete desired target genes in the neural retina.

## Introduction

The Cre-loxP system is widely used to conditionally delete target genes [1]. This allows circumventing potential embryonic lethality that would result from a constitutive deletion of essential target genes. A critical element of this system is the Cre recombinase, a nuclease encoded by the Cre gene of the bacteriophage P1 [2]. Over the last years, the Cre-loxP based technique has greatly expanded through the availability of Cre expressing mice in which expression is either controlled through cell- or tissue- specific promoter elements, or inducible by administration of certain drugs [3,4]. Still, there is growing concern that the expression of a protein originating from a different organism might itself, directly or indirectly, interfere with the cellular homeostasis of the targeted tissue or the targeted cell population. Publications showing that the expression of Cre might cause chromosomal rearrangements, growth arrest or influence gene expression profiles [5–7] provided first indications for these unwanted side effects. Furthermore, there is data showing that the expression of Cre resulted in the degeneration of neurons e.g. photoreceptor cells accompanied by an impaired neuronal function [8]. Moreover, when using drug based inducible systems, the application of drugs such as tamoxifen can have unwanted side effects, too. In support of this Kim and coworkers reported that tamoxifen exposure leads to cell death of retinal pigment epithelial (RPE) cells through caspase dependent and independent mechanisms [9].

In the current study we investigated the influence of Cre recombinase expression and topical tamoxifen exposure to neuronal tissues, with the murine retina as an easily accessible model system. For the first time, this study shows comprehensive and comparative data, addressing the retinal morphology and function, glial reactivity, the molecular expression levels of neuroprotective factors, as well as neuronal vulnerability following light induced photoreceptor degeneration. We analyzed three different, widely used transgenic Cre expressing mouse strains and compared Cre expressing or tamoxifen-exposed retinæ to control retinæ. We used the  $\alpha$ -Cre line, the *Lmop-Cre* line and the tamoxifen inducible *CAGGCre-ER<sup>TM</sup>* line [10–12], all of them having a robust expression of Cre in the entire retina, or certain cellular subpopulations of the retina. In summary, we did not observe Cre

recombinase or tamoxifen induced changes in retinal structure and function, glial reactivity, expression of neuroprotective factors or photoreceptor vulnerability in any of the models investigated.

## Material and Methods

### *Mice*

All procedures conformed to the tenets of the National Institutes of Health Guidelines on the Care and Use of Animals in Research, the EU Directive 2010/63/E, and institutional guidelines. All mice were albino, homozygous for the L450 variant of RPE65 [13] and reared in 12 h light–12 h dark cycles (lights on at 7 am). Transgenic *CAGGCre-ER<sup>TM</sup>* [11] and  $\alpha$ -Cre [10] mice were bred in a mixed FVB/N × Balb-c background (F1) and *Lmop-Cre* [12] were bred in a CD1 × Balb-c background.

### *Tamoxifen treatment*

The Cre recombinase in transgenic *CAGGCre-ER<sup>TM</sup>* is inducible. These mice carry the *Cre-ER<sup>TM</sup>* fusion protein comprised of the Cre-recombinase protein fused to a mutant form of the mouse estrogen receptor [11]. The fusion protein is restricted to the cytoplasm and *Cre-ER<sup>TM</sup>* will only access the nucleus after binding to tamoxifen. We additionally used Balb-c wildtype animals to analyze the influence of tamoxifen treatment only. To this end, tamoxifen (Cayman Chemicals, Ann Arbor, MI, USA) was diluted in corn oil (Sigma-Aldrich Chemie GmbH, Munich, Germany) to a final concentration of 5 mg/ml and the solution was topically applied as eye drops (10  $\mu$ l/drop) onto the eyes of *CAGGCre-ER<sup>TM</sup>* mice and control littermates or Balb-c wildtypes, respectively, three times per day in four hours intervals as described previously [14,15]. Our treatment started at postnatal day (P) 28 and lasted to P32. As a side note regarding the use of tamoxifen: we used tamoxifen, not 4OH-tamoxifen, to activate the Cre recombinase in *CAGGCre-ER<sup>TM</sup>* animals. Tamoxifen binds to the mutant form of the mouse estrogen receptor with lower affinity than 4OH-tamoxifen [16,17]. However, due to the topical application, very high local concentrations are reached, resulting

in a successful activation of the Cre recombinase in these animals, as can be seen in Fig. 1 and [11,14,15].

#### *PCR analyses for genotyping*

Genotypes were screened by isolating genomic DNA from tail biopsies and testing for transgenic sequences by PCR as described previously [18]. For detection of *Cre* primer sequences were 5'-ATG CTT CTG TCC GTT TGC CG-3' (sense) and 5'-CCT GTT TTG CAC GTT CAC CG-3' (antisense) and for *lacZ* genotyping the primer sequences were 5'-ATC CTC TGC ATG GTC AGG TC-3' (sense) and 5'-CGT GGC CTG ATT CAT TCC-3' (antisense). The thermal cycle profile was denaturation at 96 °C for 30 s, annealing at 57 °C (*Cre*) or 60 °C (*lacZ*) for 30 s, and extension at 72 °C for 35 s for 35 cycles.

#### *$\beta$ -galactosidase staining*

Heterozygous *CAGGCre-ER<sup>TM</sup>*,  *$\alpha$ -Cre* or *Lmop-Cre* mice were crossed with homozygous *Cre*-reporter (*R26R*) mice (genetic background: 129SV) [19]. The resulting *CAGGCre-ER<sup>TM</sup>* (or  *$\alpha$ -Cre* or *Lmop-Cre*)/*R26R* mice were used as experimental mice, and *R26R* littermates as control mice. Lac-Z-staining was performed in mixed *CAGGCre-ER<sup>TM</sup>* (or  *$\alpha$ -Cre* or *Lmop-Cre*)/*R26R* and *R26R* mice following a previously published protocol [18]. Paraffin sections (6  $\mu$ m thick) were analyzed using an Axio Imager Z1 light microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) and the appropriate Axiovision software 4.8 (Carl Zeiss Microscopy GmbH, Jena, Germany).

#### *Microscopy and morphometry*

Eyes were isolated, post-fixed for 24 h in modified Karnovsky fixative [20] and embedded in Epon (SERVA Electrophoresis GmbH, Heidelberg, Germany). Semithin sagittal sections (1.0  $\mu$ m thick) were cut through the eyes and stained after Richardson [21]. Sagittal sections were investigated using an Axio Imager Z1 fluorescent microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) and the appropriate Axiovision software 4.8 (Carl Zeiss Microscopy GmbH, Jena, Germany). Thickness of the outer nuclear layer (ONL) and inner nuclear layer (INL) were measured on semithin sections along the mid-horizontal (nasal-temporal) plane. Briefly,

the distance between ora serrata (OS) and optic nerve head (ONH) was divided into tenths to defined individual measure points. We measured one section per animal and one measurement (INL and ONL) per measure point. The sections were always stretching through the optic nerve head to ensure comparable conditions between the individual sections. Subsequently, ONL or INL thickness was measured at these measure points and the means $\pm$  SEM of the ONL or INL thickness were plotted in a spider diagram as described in [18,22].

### *Immunohistochemistry*

Prior to GFAP staining, eyes were fixed for 4 h in 4% paraformaldehyde (PFA), washed extensively in phosphate buffer (PB, 0.1 M sodium phosphate, pH 7.4) and embedded in paraffin according to standard protocols. Paraffin sections (6  $\mu$ m) were deparaffinized and washed in H<sub>2</sub>O. IBA-1 immunohistochemistry was performed on frozen sections. Eyes were fixed for 4 h in 10% glacial acetic acid, 60% methanol and 30% chloroform. Eyes were consecutively washed with 50%, 25% methanol and PB for 30 min each and incubated for several hours in each 10%, 20% and 30% sucrose/PB at 4°C. Finally, samples were rapidly frozen in tissue mounting medium (DiaTec, Bamberg, Germany). For IBA-1 immunohistochemistry, the sections were pre-treated with 0.2 M NH<sub>4</sub>Cl for 30 min to minimize potential unspecific background staining, then washed three times in PB for 5 min each and incubated in Triton-X100 for 5 min. All sections were washed three times in PB for 5 min each and blocked with 2% BSA/0.1% Triton in PB for 45 min at room temperature. Primary antibodies (GFAP (Dako Deutschland GmbH, Hamburg, Germany) 1:1000, IBA-1 (Wako Chemicals GmbH, Neuss, Germany) 1:500) were diluted in a 1:10 dilution of the blocking solution in PB and incubated at 4°C overnight. To test for the specificity of the antibody, negative control sections were incubated with the 1:10 dilution of the blocking solution only. After three 5 min PB buffer wash steps, the sections were incubated for 1 h with secondary antibodies (GFAP: anti-rabbit Alexa 546 (Thermo Fisher Scientific, Waltham, MA USA) 1:1000 or IBA-1: anti-rabbit Cy<sup>TM</sup>-3 conjugated (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) 1:2000) in blocking solution 1:10 diluted in PB.

Sections were washed three times in PB and cell nuclei were counterstained with DAPI (Vector Laboratories, Inc., Burlingame, CA, USA, final concentration of 0.15  $\mu\text{g/ml}$ ) in Moviol fluorescent mounting medium.

#### *Electroretinography*

Mice were dark adapted for at least 12 h before experiments and anesthetized by s.c. injection of ketamine (65 mg/kg) and xylazine (13 mg/kg). Pupils were dilated with tropicamide eyedrops (Mydriaticum Stulln; Pharma Stulln GmbH, Stulln, Germany). Silver needle electrodes served as reference (forehead) and ground (tail) and gold wire ring electrodes as active electrodes. Corneregel (Bausch & Lomb GmbH, Berlin, Germany) was applied to keep the eye hydrated and to maintain good electrical contact. ERGs were recorded using a Ganzfeld bowl (Ganzfeld QC450 SCX, Roland Consult, Stasche & Finger GmbH, Brandenburg a.d.Havel, Germany) and an amplifier & recording unit (RETI-Port, Roland Consult, Stasche & Finger GmbH, Brandenburg a.d.Havel, Germany). ERGs were recorded from both eyes simultaneously, band-pass filtered (1 to 300 Hz) and averaged. Single flash scotopic (dark adapted) responses to a series of ten LED-flash intensities ranging from -3.5 to 1.0 log  $\text{cd.s/m}^2$  with an inter stimulus interval of 2 up to 20 seconds for the highest intensity were recorded. After 10 minutes of adaptation to a white background illumination (20  $\text{cd/m}^2$ ) single flash photopic (light adapted) responses to three Xenon-flash intensities (1, 1.5 and 2 log  $\text{cd.s/m}^2$ ) were recorded. All analysis and plotting was carried out with R 3.2.1 (The R Foundation for Statistical Computing) and ggplot2 1.0.1 [23].

#### *Fundus imaging and angiography*

Retinal imaging was performed with a commercially available imaging system (Micron III; Phoenix Research Labs, Pleasanton, CA, USA). Light source path and imaging path filters (low pass and high pass at 500 nm) were used for fluorescein angiography (FLA). Mice were anesthetized by s. c. injection of ketamine (65 mg/kg) and xylazine (13 mg/kg), and their pupils were dilated with tropicamide eyedrops (Mydriaticum Stulln; Pharma Stulln GmbH,

Stulln, Germany) before image acquisition. FLA was performed using a s.c. injection of 75 mg/kg body weight fluorescein-Na (ALCON Pharma GmbH, Freiburg, Germany).

#### *RNA analysis*

Total RNA from neural retinae was extracted using TRIzol and first strand cDNA synthesis was performed using iScript cDNA Synthesis Kit (Bio-Rad Laboratories GmbH, Munich, Germany) according to manufacturer's instructions. Quantitative real-time RT-PCR analyses were performed using the Bio-Rad iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories GmbH, Munich, Germany). The temperature profile was denaturation at 95°C for 10 seconds and annealing and extension at 60°C for 40 seconds for 40 cycles. All primer pairs were purchased from Invitrogen and extended over exon–intron boundaries, except for *Gapdh*. Sequences of primer pairs are shown in table 1. RNA that was not reverse transcribed served as negative control for real-time RT-PCR. Three different potential housekeeping genes were tested: glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*), guanine nucleotide binding protein (*Gnb2l*), and ribosomal protein L32 (*Rpl32*). After statistical evaluation of the different housekeepers, the geo mean of the housekeeping genes was used for relative quantification of retinae samples. Quantification was performed using BioRad iQ5 Standard-Edition (Version 2.1) software (Bio-Rad Laboratories GmbH, Munich, Germany) and the  $\Delta\Delta C_t$  method in Excel (Microsoft Corporation, Redmond, WA, USA) [24].

#### *Light damage*

To experimentally induce the apoptosis of photoreceptor cells [25–27], six to seven weeks old albino mice that were homozygous for the L450 variant of RPE65 [13] were transferred to cyclic dim light (<100 lux) for 5 d followed by a dark-adapting period of 12 h. Mice were then placed in reflective cages and exposed to diffuse cool, white fluorescent light coming from the top of the cage with an intensity of 5000 lux for 30 min. Average intensity was measured on the cage floor (5000 lux). After light exposure, mice were allowed to recover first for 6 h in dim light and then brought back to normal cyclic light conditions before sacrifice. Light damage experiments were always performed in the early morning.

### *Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)*

Apoptotic cell death was analyzed by TUNEL labeling using the Apoptosis Detection System (DeadEnd Fluorometric TUNEL, Promega GmbH, Mannheim, Germany). Paraffin sections were treated following manufacturers' instructions and protocols reported previously [18]. For quantitative analysis, the number of TUNEL-positive nuclei in sagittal sections throughout the entire retina was counted using the ImageJ software program and normalized to the area of the ONL [18,28].

### *Statistical analysis*

All results are expressed as mean  $\pm$  standard error of the mean (SEM). Statistical significance was calculated by using a two-tailed Student's t-test. *P* values  $\leq$  0.05 were considered to be statistically significant.

## **Results**

To elucidate the potential influence of Cre recombinase expression or topical tamoxifen exposure on the neuronal retina, we compared four groups of mice: (1) Tamoxifen treated wildtype animals (control(TX)) were compared to their untreated wildtype littermates (control). (2) Tamoxifen treated transgenic *CAGGCre-ER<sup>TM</sup>* animals (*CAGGCre-ER<sup>TM</sup>*) were compared to equally tamoxifen treated wildtype littermates (control(TX)). (3) Transgenic  $\alpha$ -*Cre* animals ( $\alpha$ -*Cre*) were compared to wildtype littermates (control) and (4) *Lmop-Cre* animals (*Lmop-Cre*) were compared to wildtype littermates (control).

### *Cre recombinase expression and activity*

*CAGGCre-ER<sup>TM</sup>/R26R* mice carry a *Cre-ER<sup>TM</sup>* fusion protein comprised of the Cre recombinase protein fused to a mutant form of the mouse estrogen receptor [11]. The fusion protein is restricted to the cytoplasm and *Cre-ER<sup>TM</sup>* will only translocate to the nucleus after binding to tamoxifen. In transgenic  $\alpha$ -*Cre* mice, the Cre recombinase is expressed under the control of the  $\alpha$ -enhancer element of the pax6 promoter resulting in an early (embryonic day 10.5) and constitutive expression of Cre in all cells that originate from the inner layer of the optic cup [10]. In transgenic *Lmop-Cre* animals, the Cre recombinase is constitutively

expressed under the control of the murine opsin promoter [12]. Promotor activity starts with a rather weak expression from postnatal day 7, until Cre expression reaches gradually its maximum approximately at the age of six weeks [12]. We used the *R26R* Cre reporter mice to confirm and to visualize the activity of the Cre recombinase in all three analyzed transgenic Cre expressing mouse lines. In the *R26R* mouse line, a loxP-flanked DNA segment prevents the expression of a downstream *lacZ* gene. Consequently, when *R26R* mice are crossed to a Cre expressing transgenic mouse line, Cre expression leads to the removal of the loxP-flanked DNA segment resulting in the expression of the lacZ protein in all cells in which Cre had been expressed. Using  $\beta$ -galactosidase staining one can directly visualize the cell population in which Cre recombinase had been active [19]. In *CAGGCre-ER<sup>TM</sup>* Cre mice, we observed  $\beta$ -galactosidase staining, indicating lacZ reactivity, throughout the eyes of four weeks old *CAGGCre-ER<sup>TM</sup>/R26R* animals after tamoxifen treatment (Fig. 1A). Notably, in the retina all neuronal layers were stained, as was the retinal pigment epithelium and the adjacent choroid (Fig. 1A). The control(TX)/*R26R* littermates did not show any lacZ reaction in the eye, confirming the specificity of the performed  $\beta$ -galactosidase staining. When lacZ staining was performed in 10 days old transgenic  *$\alpha$ -Cre* mice, the  $\beta$ -galactosidase reaction was restricted to the neuronal layers of the retina and the non-pigmented epithelial layer of the ciliary body and iris while the control/*R26R* animal did not show any lacZ reaction (Fig. 1B). As expected, in *Lmop-Cre* animals,  $\beta$ -galactosidase staining was restricted to the outer plexiform layer, the inner and outer segments of the photoreceptors and appeared rather patchy in the outer nuclear layer at the age of three weeks (Fig. 1C). Again, the control/*R26R* animals did not show any  $\beta$ -galactosidase staining (Fig. 1C).

*Cre recombinase expression or tamoxifen treatment do not change retinal morphology and function*

To investigate, whether the expression of Cre or topical tamoxifen exposure might influence retinal morphology, we analyzed semithin section of six to ten weeks old animals. We did not observe overt structural differences in any of the three investigated transgenic Cre expressing mouse lines (*CAGGCre-ER<sup>TM</sup>*,  *$\alpha$ -Cre* line and *Lmop-Cre* line), nor in the

tamoxifen treated control(TX) animals, compared to their respective controls. All investigated eyes were well developed and showed a regular neuronal architecture (Fig. 2A, D, G, J), as well as a regular retinal vasculature, determined by *in vivo* fluorescence angiography (Fig 3A, B, C). To conduct a detailed morphometric analysis, we measured the thickness of the inner nuclear layer (INL) and the outer nuclear layer (ONL). The mean values of the thickness at individual measure points were plotted in a spider diagram followed by statistical evaluation. Here, we could not detect any statistically significant differences between experimental (Cre expressing and/or tamoxifen-treated) and control mice (INL: Fig. 2B, E, H, K; ONL: Fig. 2C, F, I, L). When investigating retinal function by ERG analysis, we focused on the *CAGGCre-ER<sup>TM</sup>* line, as this mouse line showed the most robust expression of Cre in the entire retina following tamoxifen treatment. Here, we could not detect any statistically significant differences between *CAGGCre-ER<sup>TM</sup>* animals and their control(TX) littermates (Fig. 4). ERG analyses showed similar waveforms for both, the dark and light adapted responses of the *CAGGCre-ER<sup>TM</sup>* and control(TX) animals (Fig. 4). The a- and b-wave amplitudes (Fig. 4A and B) and implicit times (Fig. 4C) were similar for all flash strengths of the dark adapted ERG responses indicating a regular rod function. Transgenic *CAGGCre-ER<sup>TM</sup>* mice showed similar waveforms (Fig. 4D) and amplitudes (Fig. 4E) compared to control(TX) littermates for the light adapted ERG responses. The small differences in implicit times (Fig. 4E) were not statistically significant, indicating a normal cone function. In summary, these findings showed that the ectopic expression of Cre or topical tamoxifen treatment neither changes retinal morphology nor do they interfere with retinal function.

*Cre recombinase expression or tamoxifen treatment do not induce macro- and microglia reactivity*

Next we investigated, whether the expression of Cre or tamoxifen exposure might cause reactive changes in the retinal microglia. To this end, we performed immunohistochemistry using an antibody against ionized calcium binding adaptor molecule 1 (IBA1) to visualize the retinal microglial cells [29]. We did not observe any obvious structural changes in the morphological appearance of the microglia cells that would indicate a switch from a resting to

a reactive phenotype [14,30,31] when comparing microglial cells of Cre expressing mice to their respective wildtype littermates. In *CAGGCre-ER<sup>TM</sup>*, *α-Cre*, *Lmop-Cre*, tamoxifen treated control(TX) animals and wildtype mice, IBA1-positive microglia cells showed a ramified morphology with the characteristic localization of these cells in the inner and outer plexiform layers and the retinal ganglion cell layer (Fig. 5A, C, D), which indicates a resting microglia cell population. Finally, we analyzed the retinae of *CAGGCre-ER<sup>TM</sup>* mice and their control littermates for markers of microglial reactivity. We restricted the analysis to the *CAGGCre-ER<sup>TM</sup>* mice as this is the only Cre line in our study, in which the microglial cells could be directly affected by the expression of Cre recombinase. However, we could not detect any statistically significant differences in the expression levels of the investigated molecular markers (*Cd68*, *Iba1*, *Egr1* and *Ccl2*) of *CAGGCre-ER<sup>TM</sup>* retinae, compared to control(TX) retinae (Fig. 5B). Furthermore, we did not observe any statistically significant differences comparing control retinae to tamoxifen treated control(TX) retinae (Fig. 5B). To exclude the possibility that expression of Cre or tamoxifen exposure would result in reactivity of Müller cells or astrocytes, we used immunohistochemical staining for glial fibrillary acidic marker protein (GFAP). Since mature retinal astrocytes do express GFAP [32,33], we observed an intense immunoreactivity for GFAP in the RGC layer, exactly where the retinal astrocytes are located. However, the overall intensity of GFAP and the astrocyte cell morphology did not differ between control or transgenic animals, indicating the resting astrocyte population [33,34]. Furthermore, we did not detect the characteristic radial stripes throughout the retina that would indicate a reactivity of Müller cells (Fig. 6A, C, E) [35]. Moreover, we used realtime RT-PCR analysis to screen the retinae for *Gfap* mRNA expression levels, but we did not detect any significant differences in its expression levels (Fig. 6B, D, F). Fig. 6B, *CAGGCre-ER<sup>TM</sup>* line, control(TX):  $1 \pm 0.35$ , *CAGGCre-ER<sup>TM</sup>*:  $1.04 \pm 0.19$  ( $p = 0.91$ ); Fig. 6D, *α-Cre* line, control:  $1 \pm 0.33$ , *α-Cre*:  $0.78 \pm 0.08$  ( $p = 0.58$ ); Fig. 6F, *Lmop-Cre* line: control:  $1 \pm 0.19$ , *Lmop-Cre*:  $0.87 \pm 0.14$  ( $p = 0.59$ ) and tamoxifen exposed control(TX) retinae, control:  $1 \pm 0.38$ , control(TX):  $0.88 \pm 0.38$  ( $n = 5/5$ ,  $p = 0.83$ ). Taken together, the ectopic expression of

Cre and/or tamoxifen exposure did not result in reactive changes of retinal macro- or microglial cells.

*Cre recombinase expression or tamoxifen treatment do not influence the expression of neuroprotective factors*

Next, we analyzed whether the expression of Cre or tamoxifen exposure might interfere with the expression levels of certain neuroprotective factors. Here we focused on leukemia inhibitory factor (*Lif*), endothelin 2 (*Edn2*) and fibroblast growth factor 2 (*Fgf2*), as these factors are well known to play a major role in retinal neuroprotective signaling cascades [36–39]. Using realtime RT-PCR analysis we could not detect any significant differences in the expression levels of the investigated factors in all three transgenic Cre mouse strains (*CAGGCre-ER<sup>TM</sup>*,  $\alpha$ -*Cre* line and *Lmop-Cre* line) compared to their control littermates (Fig. 7A, C, D). Furthermore, we did not observe any statistically significant differences when control retinae were compared with tamoxifen treated control(TX) retinae (Fig. 7B).

*Cre recombinase expression or tamoxifen treatment do not alter neuronal vulnerability*

We next examined whether the expression of Cre or tamoxifen exposure might have an influence on the vulnerability of neurons following experimentally induced damage. Here we focused on the photoreceptor cells and used the light damage as a model to induce photoreceptor degeneration through apoptosis. We labeled the apoptotic photoreceptor cells in the light exposed animals by using the 'terminal deoxynucleotidyl transferase dUTP nick end labeling' (TUNEL) method (Fig. 8). Subsequently, we counted the total number of TUNEL positive cells and normalized this number to the area of the ONL (Fig. 8C, F, I). In the following statistical evaluation, we could not detect any statistically significant differences between the transgenic lines (*CAGGCre-ER<sup>TM</sup>* line, control(TX):  $1 \pm 0.32$ , *CAGGCre-ER<sup>TM</sup>*:  $0.81 \pm 0.23$  ( $p = 0.63$ );  $\alpha$ -*Cre* line, control:  $1 \pm 0.10$ ,  $\alpha$ -*Cre*:  $1.16 \pm 0.11$  ( $p = 0.74$ ); *Lmop-Cre* line, control:  $1 \pm 0.22$ , *Lmop-Cre*:  $0.86 \pm 0.14$  ( $p = 0.66$ )), the tamoxifen exposed animals (control:  $1 \pm 0.16$ , control(TX):  $0.67 \pm 0.11$  ( $n = 4/5$ ,  $p = 0.13$ )) and the corresponding controls.

## Discussion

For the first time, our study addresses the long standing discussion whether expression of Cre recombinase or tamoxifen exposure itself might alter neuronal vulnerability. In the light of our results, we conclude that neither the ectopic expression of Cre recombinase, nor topical tamoxifen exposure via eye drops affect structure or function of the neuronal retina. This conclusion is based on the findings that neither the retinal morphology, nor function was different in control animals compared to transgenic Cre expressing animals from three different mouse lines. The same was true for the comparison of control animals to tamoxifen exposed animals. Furthermore, no changes were observed in macro- and microglial cell reactivity and glial marker expression patterns. Finally, we could neither detect differences in the mRNA expression levels of several neuroprotective factors, nor did we detect any changes in photoreceptor vulnerability, whether Cre recombinase or tamoxifen was present or not.

Our study characterizes potential side effects of Cre expression or its nuclear translocation, in the case of the CAGGCre-ER<sup>TM</sup> mouse, to the retina. Formally, we cannot exclude potential side effects arising of a Cre-mediated recombination in the genomic DNA. Our results regarding retinal morphology and morphometry are in line with previously reported observations by Le and coworkers, who also showed that these parameters were not altered in *Lmop-Cre* animals [40]. However, we included two additional transgenic mouse lines with an even more pronounced retinal expression of Cre: the  $\alpha$ -Cre [10] and the tamoxifen inducible CAGGCre-ER<sup>TM</sup> mouse line [11] and characterized all lines in a comprehensive and comparative way. Additionally, we characterized the retinal vasculature using *in vivo* fluorescence angiography and analyzed the expression profiles of molecular markers and changes in photoreceptor vulnerability for all three mouse lines and after tamoxifen treatment. Furthermore, we studied the potential effects on glial cells in the retina.

Neither tamoxifen exposure, nor the presence of Cre recombinase led to any detectable changes in reactivity of macro- or microglial cells, important components of the retinal innate

immune system [30,35,41–43]. In conclusion, Cre recombinase, an exogenous protein, does not provide an immunological challenge if translated in mouse cells. Müller cell reactivity or gliosis is one of the earliest and most sensitive markers of retinal damage [35,44]. The absence of typical parameters of the Müller cell gliosis reaction like an increase in GFAP expression strongly supports the concept that the retinal presence of Cre recombinase and/or tamoxifen exposure do not interfere with structure and function of retinal cells. However, the eye is an immune privileged organ with a high level of transforming growth factor (TGF)- $\beta$ 2, contributing to the quiescence of microglia and Müller cells [30,42,43,45,46]. This raises the possibility that the high level of TGF- $\beta$ 2 counteracts a potential cellular reactivity of these cells upon the ectopic expression of Cre or tamoxifen exposure. Consequently, we cannot exclude the possibility that this situation might be different in other, less immune-privileged organs. Our group and others recently showed that upon Müller cells reactivity, as evidenced by elevated expression levels of *Gfap*, the mRNA levels of neuroprotective factors like *Edn2*, *Lif* and *Fgf2* were induced in the retina [27,44,47–49]. In this study, however, we did not observe changes in *Gfap* levels and conclusively we could also not detect any statistically significant differences in the expression of these factors, whether Cre or tamoxifen exposure was present or not.

The expression of Cre recombinase might pose especially problematic for photoreceptors, because they are more prone to become apoptotic as soon as their cellular homeostasis is disturbed. This could be due to genetic disease mutations [50,51], or the expression of a foreign protein like Cre. Moreover, there is accumulating evidence that the expression of mutated or exogenous proteins in glial cells, resulting in dysregulated glial cell function, can increase the vulnerability of neuronal cells (see for example [52–54]). This gives rise to the possibility that the ectopic expression of Cre in neurons or glial cells might also increase the vulnerability of neurons. To test this possibility, we used the light damage model to induce neuronal apoptosis of photoreceptor cells [25,27,55,56]. Our data shows no statistically significant differences in the number of apoptotic neurons after light induced neuronal damage, independent of the presence of Cre.

There is evidence in the literature that tamoxifen treatment itself has unwanted side effects, e.g. cell death of retinal pigment epithelial (RPE) cells through caspase dependent and independent mechanisms [9]. Based on our results, we can exclude such potential side effects regarding an altered neuronal vulnerability, at least for the tamoxifen concentration and the application route used in our study.

In summary, we conclude that the Cre-loxP system and its induction through tamoxifen is a safe and reliable method to delete desired target genes in the neuronal retina. Neither the expression of Cre, nor tamoxifen exposure itself, interferes with the maintenance of the neuronal tissues regarding neuronal morphology, function, the expression of molecular markers and neuronal vulnerability, as well as the reactivity of macro- and microglia cells. Our study might therefore reassure neuroscientists in using this method in the future. We also thoroughly characterized three widely used Cre lines, *α-Cre*, *Lmop-Cre* and the tamoxifen inducible *CAGGCre-ER<sup>TM</sup>*, which is a valuable resource for other scientists trying to find a Cre line with Cre expression patterns suitable for their specific research needs.

## Acknowledgements

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## Figure legends

**Figure 1: Expression pattern and activity of the Cre recombinase in *CAGGCre-ER<sup>TM</sup>*, *α-Cre* and *Lmop-Cre* mice.** **A.** The intense β-galactosidase staining (upper right panel) throughout the eye in a four weeks old *CAGGCre-ER<sup>TM</sup>/R26R* mouse indicates a successful activation of Cre recombinase expression in ocular tissue following treatment with tamoxifen from postnatal day (P) 28-32. Scale bars correspond to 500 μm. The higher magnification of the β-galactosidase reaction (lower right panel) shows a homogenous staining in the retina and choroid of a four weeks old *CAGGCre-ER<sup>TM</sup>/R26R* mouse. The control littermate (control/R26R) does not show a positive reaction (left panel). Scale bars correspond to 50 μm. **B.** The intense β-galactosidase staining (upper right panel) indicates the activity of the Cre recombinase in the neuronal retina and the non-pigmented epithelium of the ciliary body (arrows) in the eye of a ten days old *α-Cre/R26R* mouse. Scale bars correspond to 200 μm. The higher magnification of the β-galactosidase stained retina (lower right panel) shows an intense reaction in the neuronal layers of the retina in a ten days old *α-Cre/R26R* mouse. The control littermate (control/R26R) does not show a positive reaction (left panel). Scale bars correspond to 50 μm. **C.** The intense β-galactosidase staining (upper right panel) in the eye of a three weeks old *Lmop-Cre/R26R* mouse indicates the activity of the Cre recombinase in the ONL. Scale bars correspond to 200 μm. The detailed magnification of the β-galactosidase reaction (lower right panel) shows a staining in the outer plexiform layer, ONL and the inner and outer segments of the photoreceptor cells of a three weeks old *Lmop-Cre/R26R* mouse. The control littermate (control/R26R) does not show a positive reaction (left panel). Scale bars correspond to 50 μm. RGC. retinal ganglion cells; INL. inner nuclear layer; ONL. outer nuclear layer; RPE. retinal pigment epithelium.

**Figure 2: Retinal morphology and morphometric analysis of the INL and ONL in *CAGGCre-ER<sup>TM</sup>*, *α-Cre*, *Lmop-Cre* and control(TX) mice.** **A.** Richardson stained semithin sections of an eight weeks old control(TX) (left) and its *CAGGCre-ER<sup>TM</sup>* (right) littermate retina. The retinal morphology does not show any obvious changes in the transgenic animal. **B./C.** Spider-diagrams reflecting the thickness of the INL (B) and ONL (C) of eight weeks old

control(TX) and *CAGGCre-ER<sup>TM</sup>* animals. Tamoxifen treatment postnatal day (P) 28-32. Data are shown as mean  $\pm$  SEM,  $n = 5/4$ ,  $p > 0.05$  (not significant), Student's t-test. **D.** Richardson stained semithin section of a 6 weeks old control (left) and its  $\alpha$ -*Cre* (right) littermate retina. The retinal morphology does not show any obvious changes in the transgenic animal. **E./F.** Spider-diagrams reflecting the thickness of the INL (E) and ONL (F) of six weeks old control and  $\alpha$ -*Cre* animals. Data are shown as mean  $\pm$  SEM,  $n = 7/5$ ,  $p > 0.05$  (not significant), Student's t-test. **G.** Richardson stained semithin section of a ten weeks old control (left) and its *Lmop-Cre* (right) littermate retina. The retinal morphology does not show any obvious changes in the transgenic animal. **H./I.** Spider-diagrams reflecting the thickness of the INL (H) and ONL (I) of ten weeks old control and *Lmop-Cre* animals. Data are shown as mean  $\pm$  SEM,  $n = 4/3$ ,  $p > 0.05$  (not significant), Student's t-test. **J.** Richardson stained semithin section of a six weeks old control (left) and an eight weeks old tamoxifen treated control(TX) (right) retina. The retinal morphology does not show any obvious changes in the tamoxifen exposed animal. **K./L.** Spider-diagrams reflecting the thickness of the INL (K) and ONL (L) of control and tamoxifen treated animals. Tamoxifen treatment postnatal day (P) 28-32. Data are shown as mean  $\pm$  SEM,  $n = 5/5$ ,  $p > 0.05$  (not significant), Student's t-test. RGC. retinal ganglion cells; INL. inner nuclear layer; ONL. outer nuclear layer.

**Figure 3: *In vivo* angiography of *CAGGCre-ER<sup>TM</sup>*,  $\alpha$ -*Cre*, *Lmop-Cre* and control(TX) mice.** *In vivo* funduscopy and fluorescence angiography of six weeks old *CAGGCre-ER<sup>TM</sup>* (**A**)  $\alpha$ -*Cre* (**B**) and *Lmop-Cre* (**C**) mice. The fundus is regular and the retinal vessels are well perfused with fluorescein (green) in the transgenic animals and not showing any obvious alterations compared to their respective controls. The same is true when control(TX) animals (panel A, left side) are compared to controls (panel B, left side). Tamoxifen treatment postnatal day (P) 28-32.

**Figure 4: *In vivo* electrography (ERG) analysis of *CAGGCre-ER<sup>TM</sup>* mice.** Dark adapted ERGs in six weeks old transgenic *CAGGCre-ER<sup>TM</sup>* mice show similar waveforms (**A**) and a- and b-wave amplitudes and implicit times (**B, C**) compared to control(TX) indicating a regular

rod function. After light adaptation again transgenic *CAGGCre-ER<sup>TM</sup>* mice show similar waveforms (D) and amplitudes (E) compared to wildtype littermates. Since the small implicit times difference (E) was not significant, our results indicate also a normal cone functions. Tamoxifen treatment postnatal day (P) 28-32.

**Figure 5: Microglia reactivity in the retinae of *CAGGCre-ER<sup>TM</sup>*, *α-Cre*, *Lmop-Cre* and control(TX) mice.** Immunoreactivity for IBA1 (red) in the retina at six weeks of age. Distinct immunoreactivity for IBA1 (arrowheads) is detectable at the inner retinal surface and inner and outer plexiform layers of the *CAGGCre-ER<sup>TM</sup>* (A), *α-Cre* (C) and *Lmop-Cre* (D) and corresponding controls/control(TX). Nuclei are DAPI-stained (blue). The antibody control sections indicate the specificity of the immunohistochemical reaction. Tamoxifen treatment postnatal day (P) 28-32. RGC. retinal ganglion cell layer; INL. inner nuclear layer; ONL. outer nuclear layer; RPE. retinal pigment epithelium. **B.** Real-time RT-PCR for *Cd68*, *Iba1*, *Egr1* and *Ccl2* mRNA in six weeks old *CAGGCre-ER<sup>TM</sup>* and corresponding control(TX) retinae and six to eight weeks old control and tamoxifen treated control(TX) retinae. Tamoxifen treatment postnatal day (P) 28-32. Data are means ± SEM. B: control(TX) n > 5, *CAGGCre-ER<sup>TM</sup>* n > 5, except for *Cd68* n=3. control n ≥ 4, control(TX) n ≥ 4, except for *Ccl2* n=3. *p* > 0.05 (not significant), Student's t-test. AU: arbitrary unit.

**Figure 6: GFAP in the retinae of *CAGGCre-ER<sup>TM</sup>*, *α-Cre*, *Lmop-Cre* and control(TX) mice.** Immunoreactivity for GFAP (red) in the retina at six weeks of age. Distinct immunoreactivity for GFAP (arrowheads) is detectable at the inner retinal surface of the *CAGGCre-ER<sup>TM</sup>* (A), *α-Cre* (C) and *Lmop-Cre* (E) and corresponding controls. Nuclei are DAPI-stained (blue). The antibody control sections indicate the specificity of the immunohistochemical reaction. Tamoxifen treatment postnatal day (P) 28-32. RGC. retinal ganglion cell layer; INL. inner nuclear layer; ONL. outer nuclear layer; RPE. retinal pigment epithelium. **B./D./F.** Real-time RT-PCR for *Gfap* mRNA in six weeks old *CAGGCre-ER<sup>TM</sup>* (B), *α-Cre* (D) and *Lmop-Cre* (F) and corresponding control retinae. Tamoxifen treatment postnatal day (P) 28-32. Data are means ± SEM. (B) control(TX) n = 5, *CAGGCre-ER<sup>TM</sup>* n=

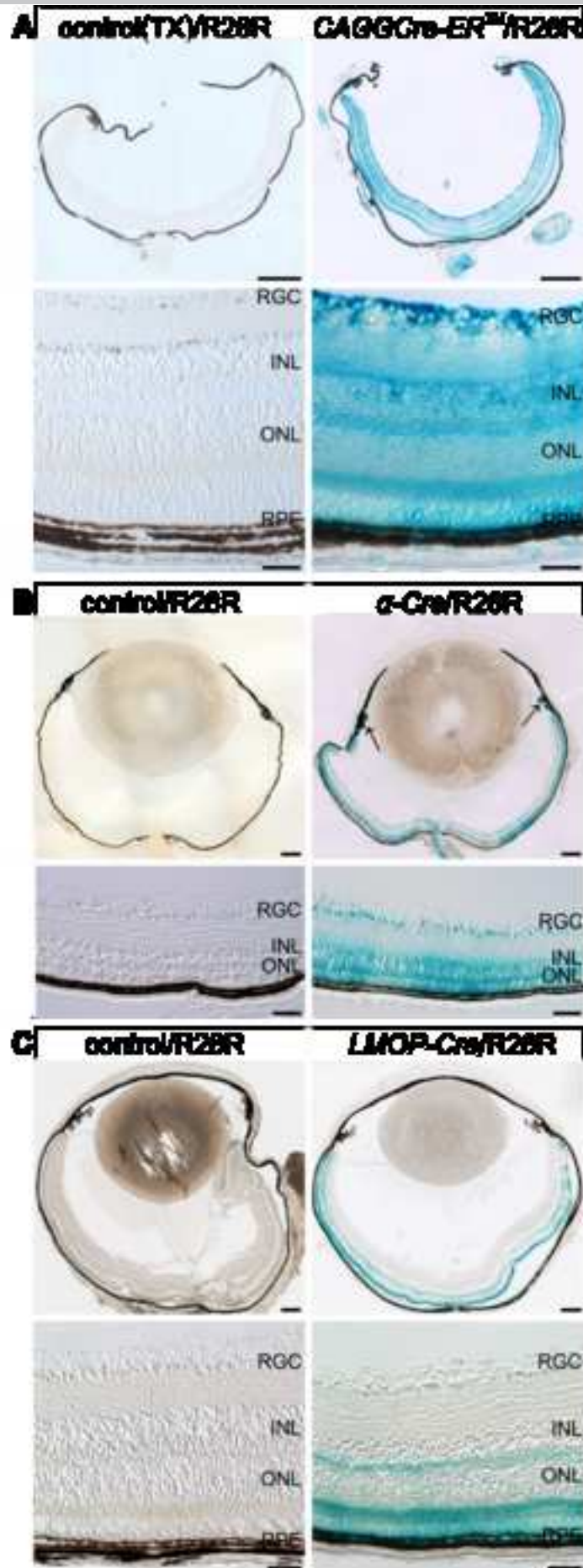
7; (D) control n = 6,  $\alpha$ -Cre n = 6; (F) control n = 4, *Lmop-Cre* n = 4; .  $p > 0.05$  (not significant), Student's t-test. AU: arbitrary unit.

**Figure 7: Expression levels of neuroprotective factors in the retinae of *CAGGCre-ER<sup>TM</sup>*,  $\alpha$ -Cre, *Lmop-Cre* and control(TX) mice.** Real-time RT-PCR for *Lif*, *Edn2* and *Fgf2* mRNA in six to eight weeks old *CAGGCre-ER<sup>TM</sup>* (A), control(TX) (B),  $\alpha$ -Cre (C) and *Lmop-Cre* (D) and corresponding control retinae. Tamoxifen treatment postnatal day (P) 28-32. Data are means  $\pm$  SEM. (A) control n  $\geq$  4, *CAGGCre-ER<sup>TM</sup>* n  $\geq$  5; (B) control n =5, control(TX) n =5 ; (C) control n = 6,  $\alpha$ -Cre n = 6; (D) control n  $\geq$  3 , *Lmop-Cre* n = 4, except for *Lif*: n = 2.  $p > 0.05$  (not significant), Student's t-test. AU: arbitrary unit.

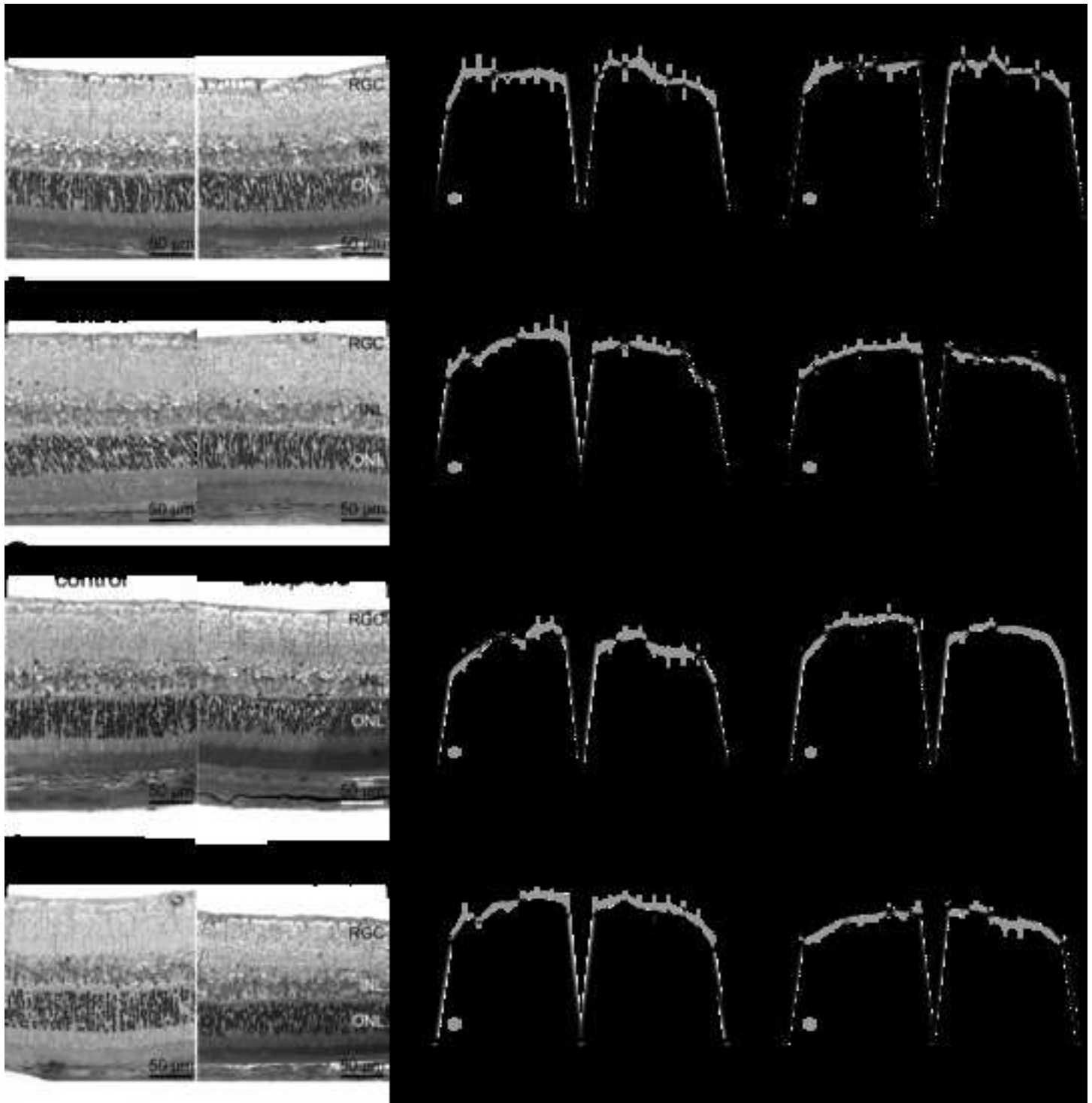
**Figure 8: Neuronal vulnerability of *CAGGCre-ER<sup>TM</sup>*,  $\alpha$ -Cre, *Lmop-Cre* and control(TX) mice following light exposure.** TUNEL labeling (green) in six weeks old retinae 30h after light exposure. The images of the retinal hemispheres show the characteristic accumulation of TUNEL positive cells in the central parts of light exposed retinae (A./D./G.) The detailed magnifications (B./E./H.) of the central retinae show the localization of the TUNEL-positive cells (arrowheads) in the ONL. Nuclei are DAPI-stained (blue). OS. Ora serrata; ON. Optic nerve; INL. inner nuclear layer; ONL. outer nuclear layer; C./F./I. Number of TUNEL-positive cells/mm<sup>2</sup> ONL in light exposed *CAGGCre-ER<sup>TM</sup>* (C),  $\alpha$ -Cre (F) and *Lmop-Cre* (I) and corresponding control retinae. Tamoxifen treatment postnatal day (P) 28-32. Data are mean  $\pm$  SEM. (C) control: n = 4, *CAGGCre-ER<sup>TM</sup>*: n = 5,  $p = 0.63$ ; (F) control: n = 7,  $\alpha$ -Cre: n = 5,  $p = 0.73$ ; (I) control: n = 2, *Lmop-Cre* n = 2,  $p = 0.66$ . Student's t-test. AU: arbitrary unit.

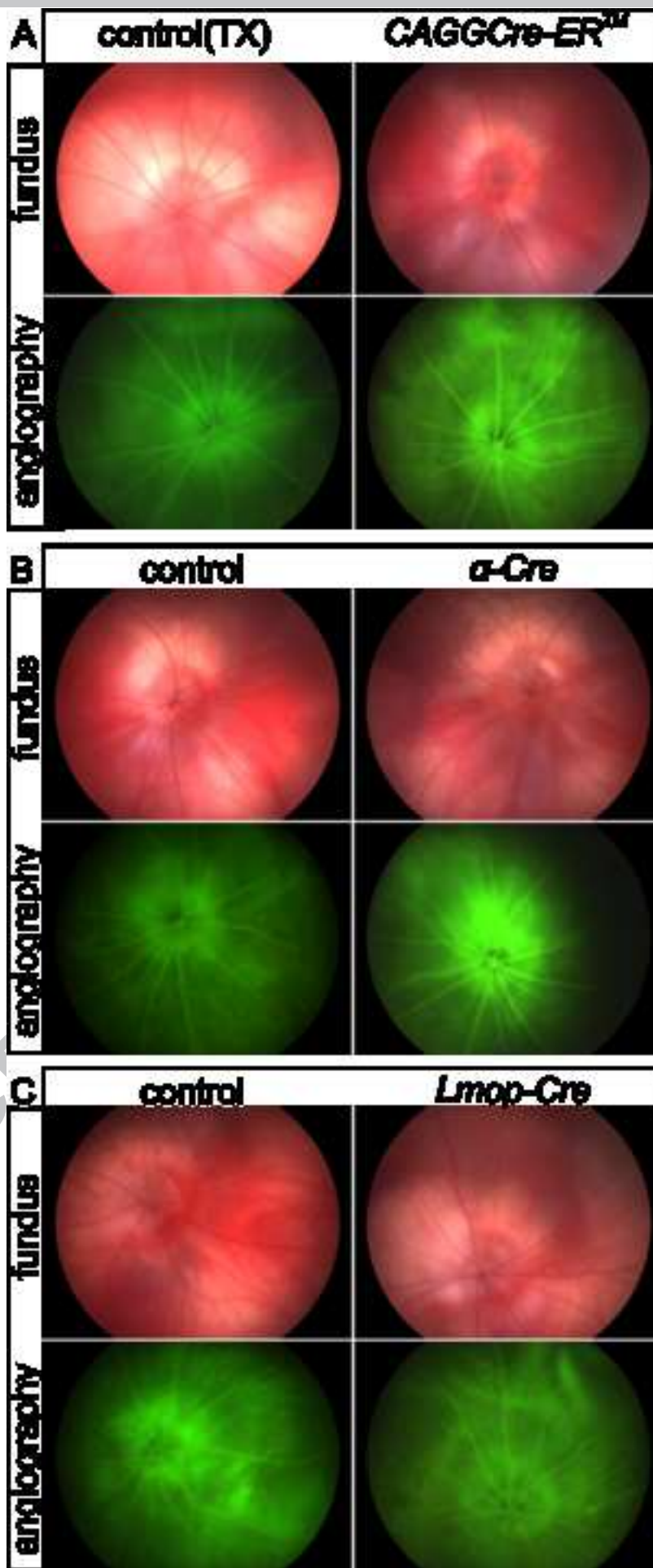
Table 1. Primers used for quantitative real-time PCR amplification

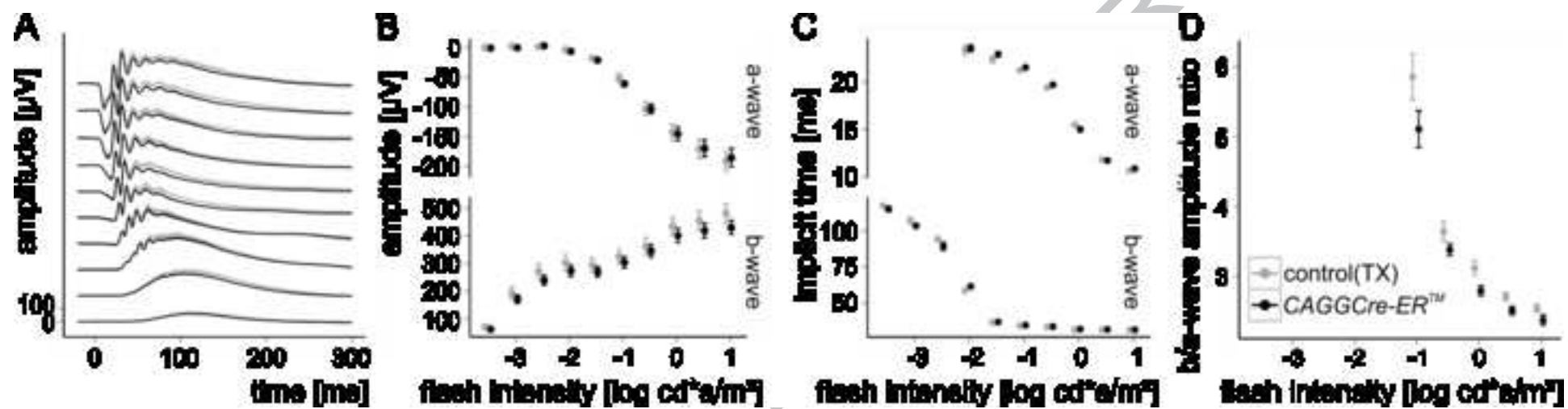
<b>Gene</b>	<b>Sequence forward</b>	<b>Sequence reverse</b>
<i>Gapdh</i>	5'-TGTCCGTCGTGGATCTGAC-3'	5'-CCTGCTTCACCACCTTCTTG-3'
<i>Gnb2l</i>	5'-TCTGCAAGTACACGGTCCAG-3'	5'-ACGATGATAGGGTTGCTGCT-3'
<i>Rpl32</i>	5'-GCTGCCATCTGTTTTACGG-3'	5'-TGA CTGGTGCCTGATGAACT-3'
<i>Lif</i>	5'-AAACGGCCTGCATCTAAGG-3'	5'-AGCAGCAGTAAGGGCACAAT-3'
<i>Edn2</i>	5'-ACCTCCTCCGAAAGCTGAG-3'	5'-TTTCTTGTACCTCTGGCTGTA-
<i>Fgf2</i>	5'-CCAACCGGTACCTTGCTATG-3'	5'-
<i>Ccl2</i>	5'-CATCCACGTGTTGGCTCA-3'	5'-
<i>Cd68</i>	5'-	5'-TCACGGTTGCAAGAGAAACA-3'
<i>Egr1</i>	5'-CCTATGAGCACCTGACCACA-3'	5'-TCGTTTGGCTGGGATAACTC-3'
<i>Iba1</i>	5'-GGATTTGCAGGGAGGAAAAG-	5'-TGGGATCATCGAGGAATTG-3'
<i>Gfap</i>	5'-TCGAGATCGCCACCTACAG-3'	5'-GTCTGTACAGGAATGGTGATGC-

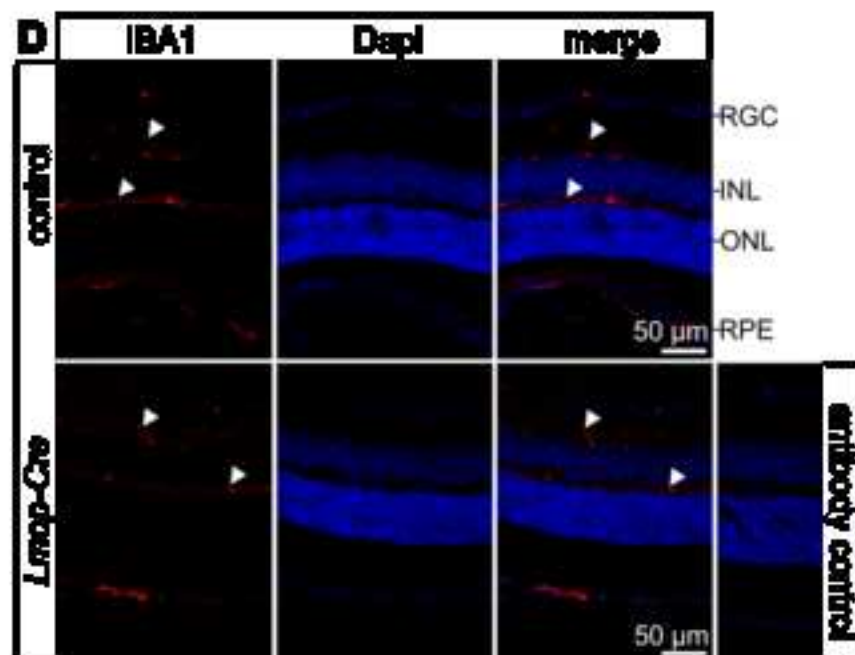
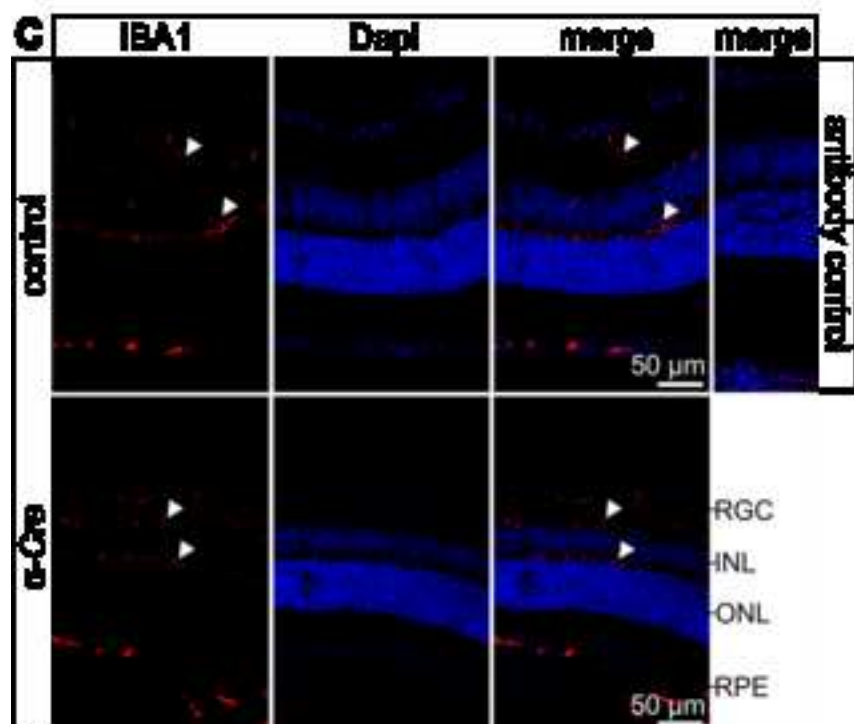
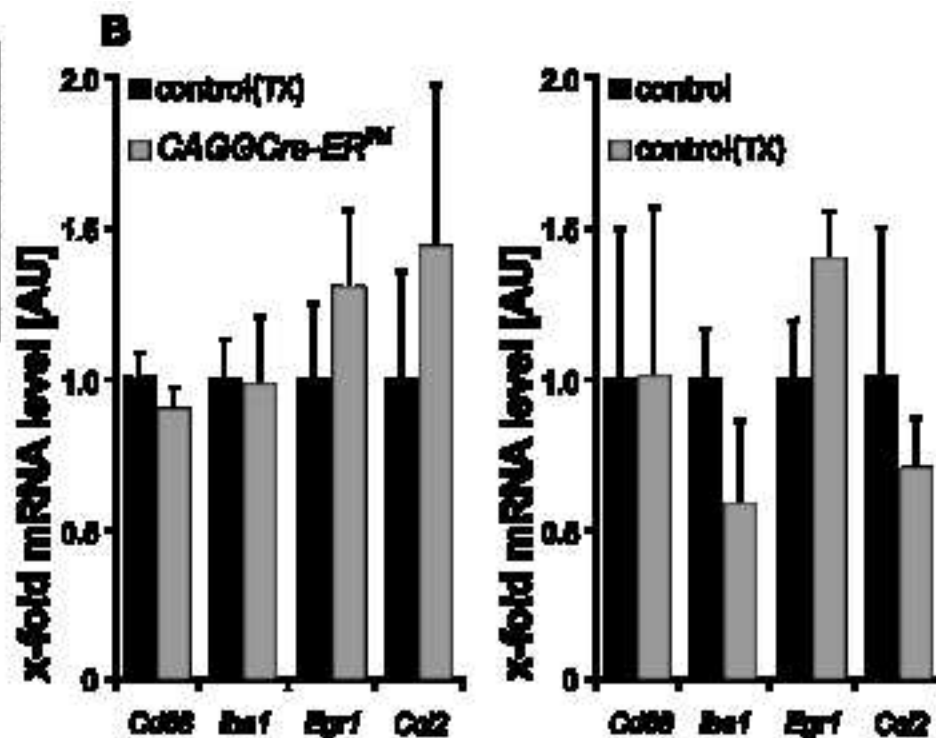
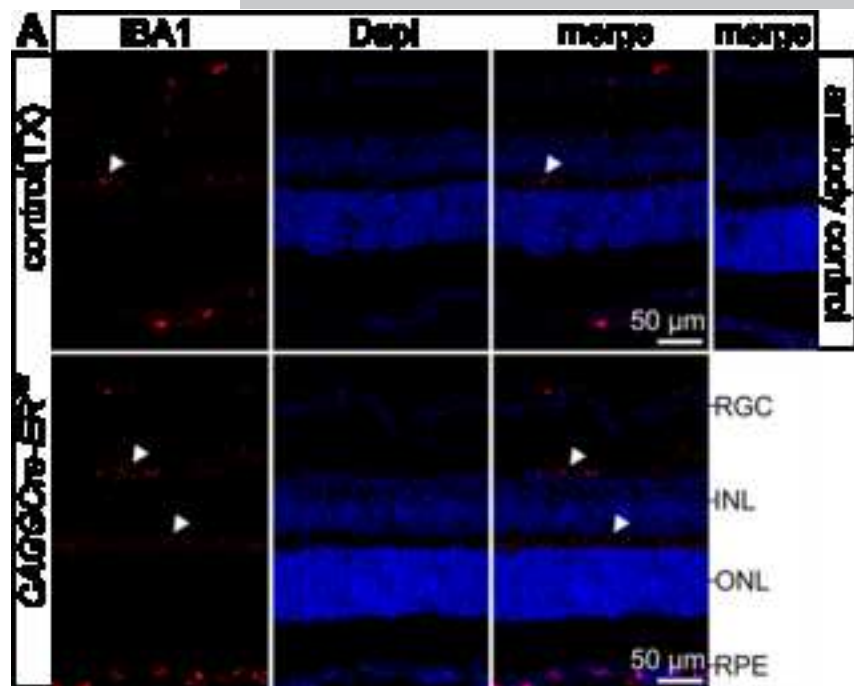


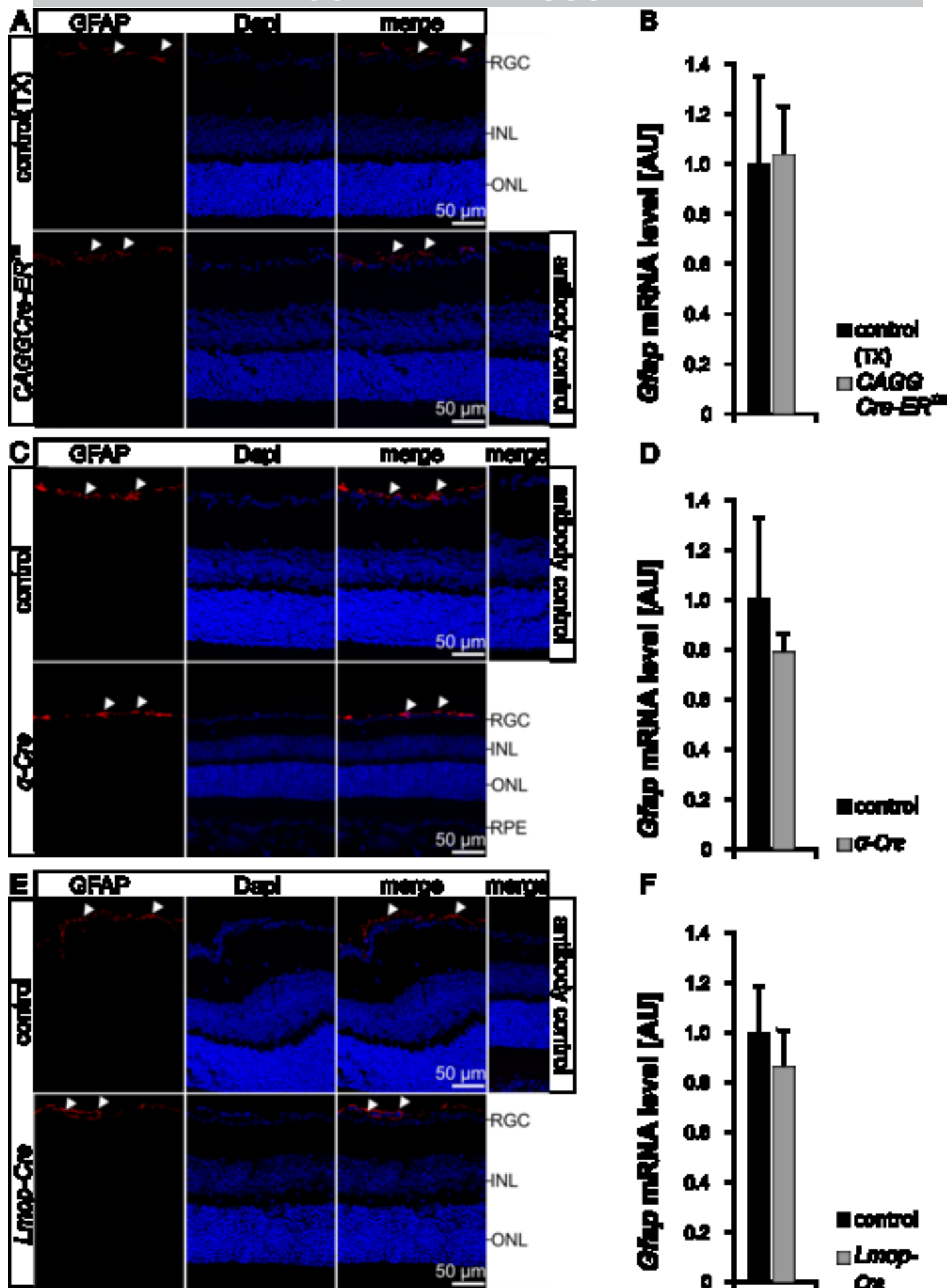
ACC

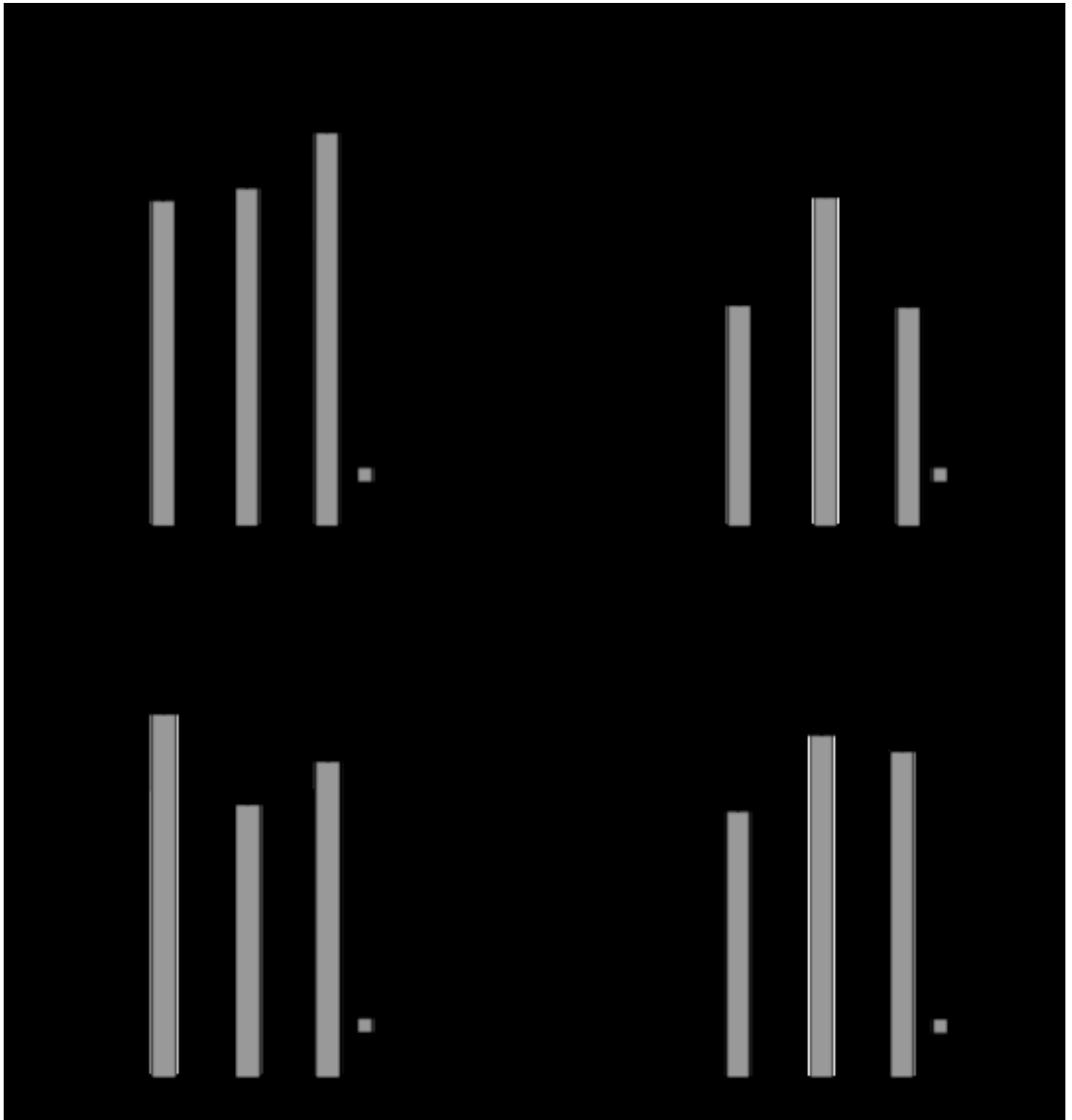


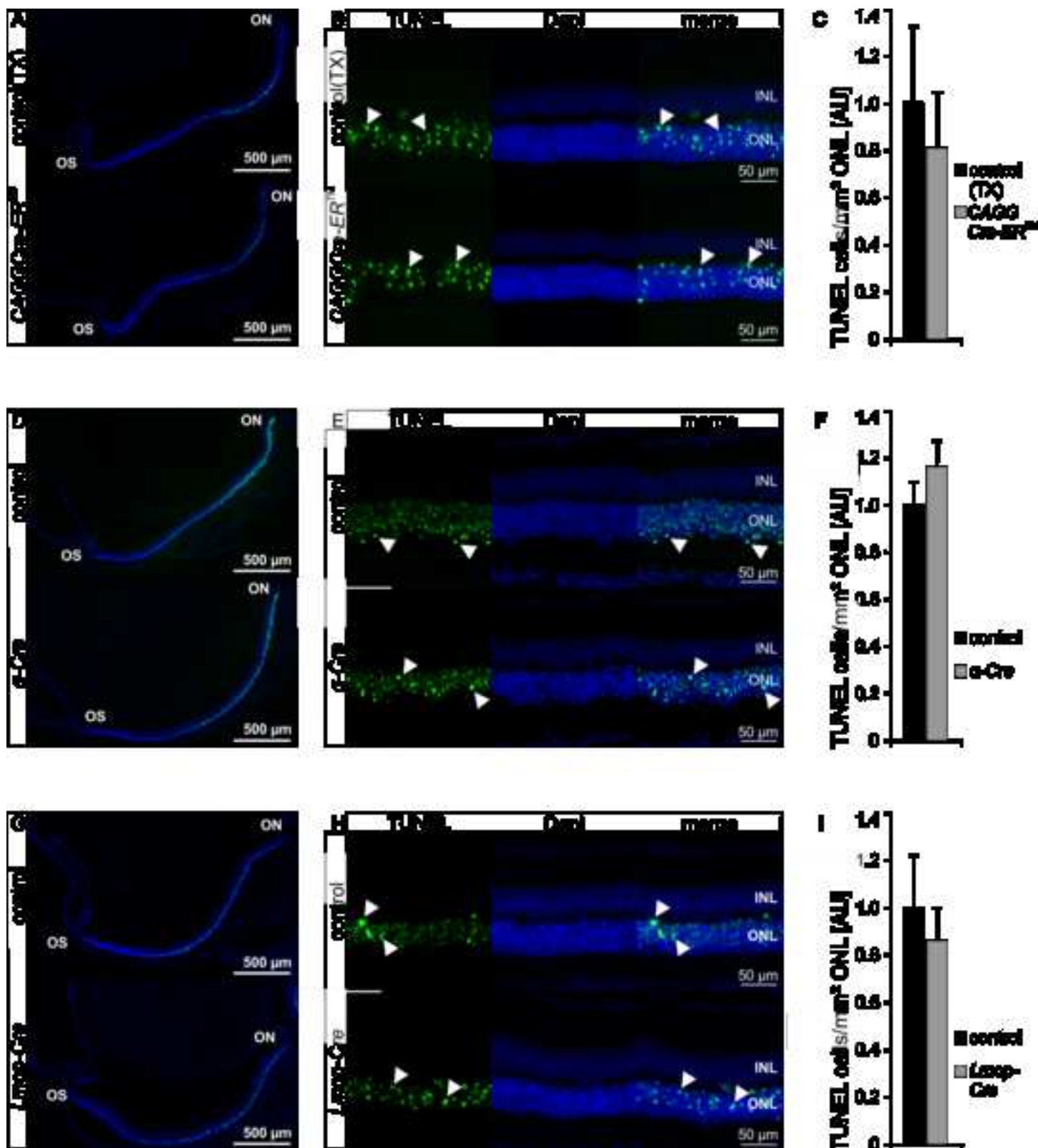












**Cre recombinase expression or topical tamoxifen treatment do not affect retinal structure and function, neuronal vulnerability or glial reactivity in the mouse eye**

Highlights:

- Cre or tamoxifen do not influence neuronal morphology and function
- Cre or tamoxifen do not induce macro- and microglia cell reactivity
- Expression of neuroprotective factors is not influenced by Cre or tamoxifen
- Cre or tamoxifen do not alter neuronal vulnerability