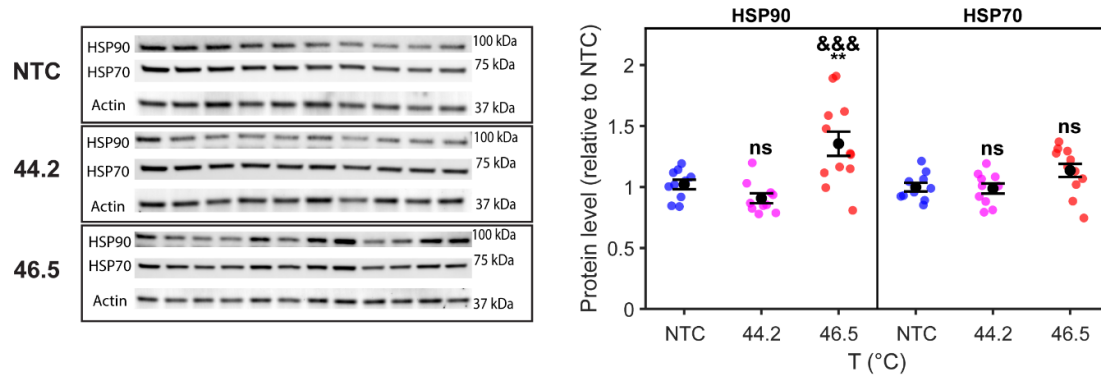
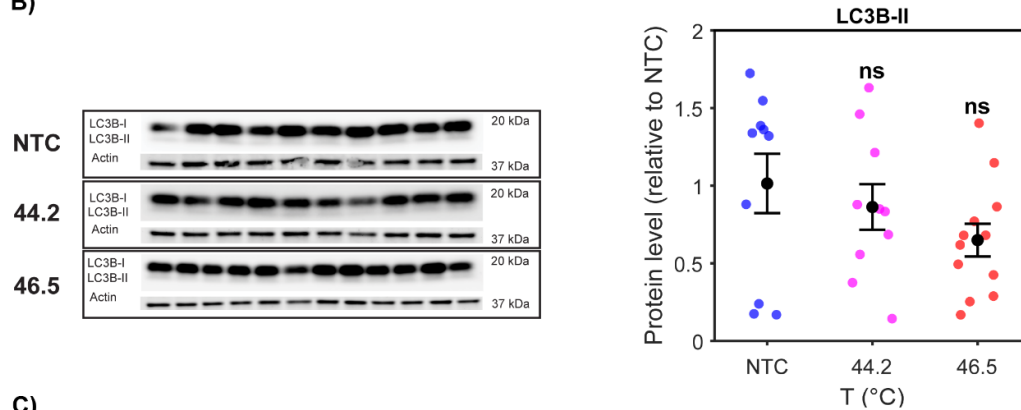


Supplementary Information

A)



B)



C)

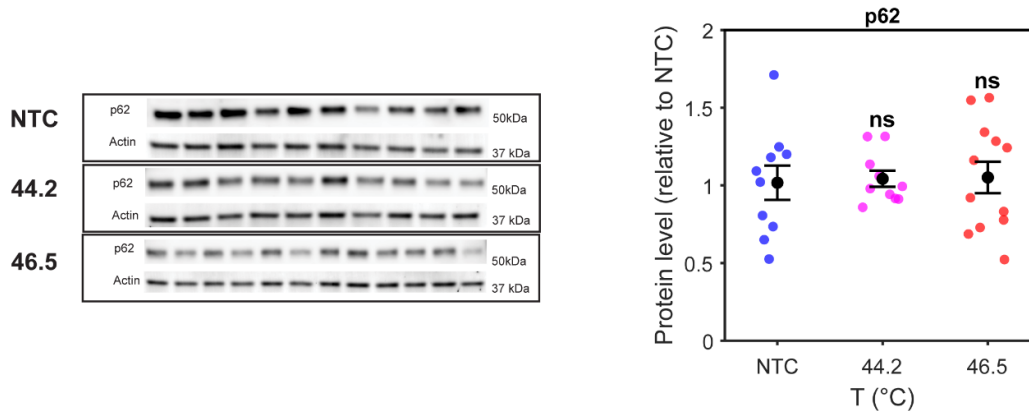


Figure S1. Heat shock induces heat shock response in the neural retina cells and has no effect on their autophagy activation.

Immunoblot analysis of **A)** HSP90 and HSP70 and **B)** LC3-II and p62 for neural retina cells 24 h after laser treatment is illustrated with representative images. Quantitative analysis of protein level was corrected to actin as the loading control. All samples were processed concurrently on the same gel. Data are represented as mean $\pm$ SEM (n = 10 for NTC, n=10 for the 44.2 °C group, n=12 for the 46.5 °C group). Statistical analysis used was one-way ANOVA followed by Bonferroni's multiple comparisons (ns; non-significant, ** p < 0. 01 vs. NTC, &&& p < 0.001 vs. the 44.2 °C group). NTC stands for non-treated control and temperatures (T) 44.2 °C and 46.5 °C represent group mean values with a standard deviation of 0.3 °C. Source data are provided as a Supplementary Information Source Data file.

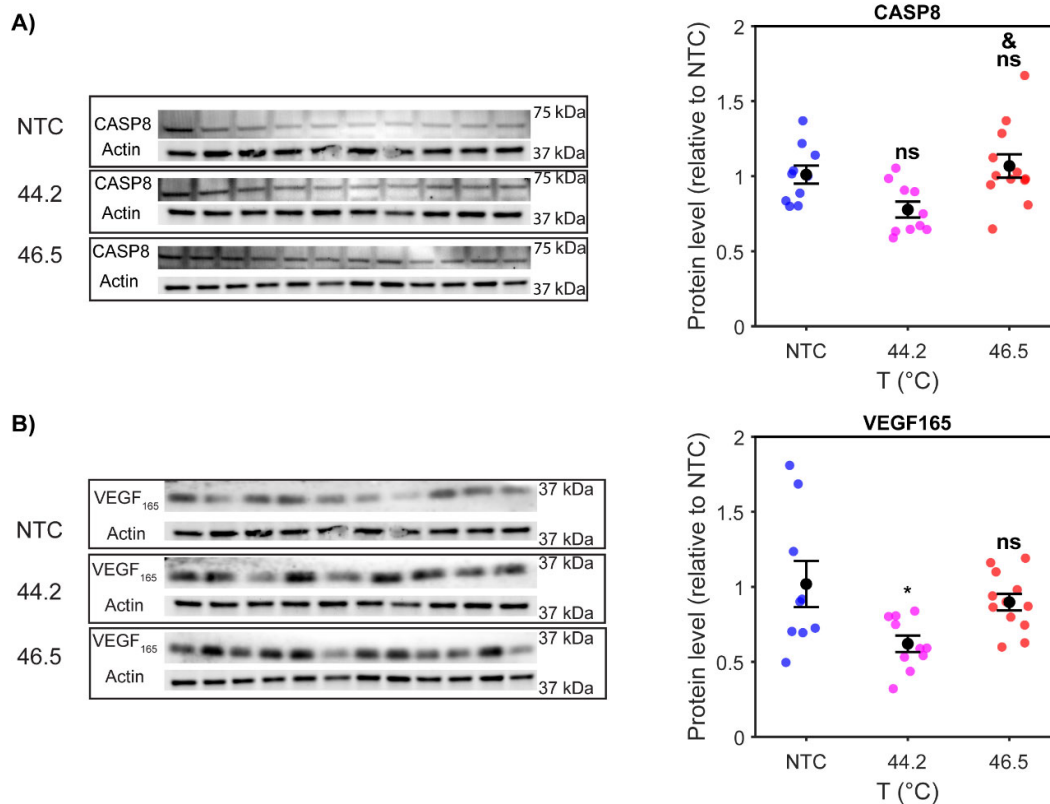


Figure S2. Induced heat shock in the neural retina cells has no effect on the activation of apoptosis while heating the retina to 44.2 °C seems to decrease the VEGF level

Immunoblot analysis of **A)** CASP8 and **B)** VEGF-165 for neural retina cells 24 h after laser treatment is illustrated with representative images. Quantitative analysis of protein level was corrected to actin as the loading control and normalized to NTC. All samples were processed concurrently on the same gel. Data are represented as mean $\pm$ SEM ($n = 10$ for NTC, $n=10$ for the 44.2 °C group, $n=12$ for the 46.5 °C group). Statistical analysis used was one-way ANOVA followed by Bonferroni's multiple comparisons (ns; non-significant, * $p < 0.05$ vs. NTC, & $p < 0.05$ vs. the 44.2 °C group). NTC stands for non-treated control. And temperatures (T) 44.2 °C and 46.5 °C represent group mean values with a standard deviation of 0.3 °C. Source data are provided as a Supplementary Information Source Data file.

Table S1. Complete list of analyzed genes, fold changes, and p-values from two-sided unpaired t-tests comparing expression levels in laser-heated versus control samples. Source data are provided as a Supplementary Information Source Data file.

#	Symbol	Description	GeneBank	Fold change 44.2 °C	p-value	Fold change 46.5 °C	p-value
Heat stress							
1	CRYAB	Crystallin, alpha B	XM_003357294	0.64	0.3161	0.57	0.2872
2	HSF1	Heat shock transcription factor 1	NM_001243819	3.71	0.0419	3.81	0.0491
3	HSF2	Heat shock transcription factor 2	XM_005659227	26.73	0.0372	19.66	0.0246
4	HSP90	Hsp90 co-chaperon cdc37-like 1- like	XM_003121918	17.84	0.0001	8.22	0.0332
5	HSP70	Heat shock protein 70	NM_001123127	6.83	0.0383	3.55	0.0162
6	HSP27	Heat shock 27 kDa protein 1	NM_001007528	1.38	0.3322	1.31	0.5076

7	HSP60	Heat shock 60 kDa protein 1 (chaperonin)	NM_001254716	0.91	0.7884	0.39	0.0294
8	HSP 40	DnaJ (Hsp40) homolog, subfamily B, member 12	XM_001924319	1.46	0.7775	0.06	0.2534
Autophagy							
9	ATG5	ATG5 autophagy related 5 homolog (S.cerevisiae)	NM_001037152	10.02	0.0472	3.76	0.032
10	ATG7	ATG7 autophagy related 7 homolog (S.cerevisiae)	NM_001190285	14.93	0.0016	6.45	0.0134
11	ATG3	Autophagy related 3	XM_003132682	18.79	0.3286	1.23	0.7835
12	BNIP3L	BCL2/adenovirus E1B 19kDa interacting protein 3-like	XM_001927592	174.78	0.0813	0.72	0.7457
13	BECN1	Beclin 1, autophagy related	NM_001044530	2.34	0.0414	0.88	0.7874
14	DRAM1	DNA-damage regulated autophagy modulator 1	NM_001190286	3.95	0.1724	0.31	0.0489
15	DRAM2	DNA-damage regulated autophagy modulator 2	NM_001243696	4.59	0.095	2.77	0.1945
16	LAMP2A	Lysosomal-associated membrane protein 2	NM_001244255	16.23	0.0385	0.53	0.4927
17	LAMP-1	Lysosomal-associated membrane glycoprotein 1	NM_001011507	1.32	0.6	1.06	0.8932
18	MAP1LC3A	Microtubule-associated protein 1 light chain 3 alpha	NM_001170827	0.66	0.319	0.95	0.8985
19	MAP1LC3B	Microtubule-associated protein 1 light chain 3 beta	NM_001190290	21.44	0.0185	8.37	0.0111
20	MTOR	Mechanistic target of rapamycin (serine/threonine kinase)	XM_003127584	0.76	0.6996	1.16	0.8221
21	SQSTM1	Sequestome-1-like	XM_003123639	3.99	0.1992	0.18	0.2985
22	TGFB1	Transforming growth factor, beta 1	NM_214015	0.56	0.3277	0.18	0.0275
23	TLR9	Toll-like receptor 9	NM_213958	2.43	0.158	0.55	0.3275
24	UBQLN1	Ubiquilin 1	XM_021064658	0.46	0.3342	0.31	0.1729
25	ULK1	Unc-51-like kinase 1 (C.elegans)	XM_005670526	3.42	0.0414	1.35	0.6935
26	ULK2	Unc-51-like autophagy activating kinase 2	XM_013981364	22.63	0.0222	2.97	0.0167
Oxidative Stress							
27	GPX1	Glutathione peroxidase 1	NM_214201	3.16	0.0383	2.94	0.0496
28	GSS	Glutathione synthetase	NM_001244625	2.10	0.1256	1.50	0.1256
29	Hmox1	Heme Oxygenase (decycling) 1	NM_001004027	0.73	0.4519	1.13	0.5804
30	Nos2	Nitric oxide synthase 2, inducible	NM_001143690	1.48	0.4489	0.60	0.3857
31	Sod1	Nitric oxide synthase 1 (neuronal)	XM_013990334	3.36	0.0239	2.54	0.1763
32	Sod2	Nitric oxide synthase 1 (neuronal)	XM_013990334	2.74	0.0968	3.32	0.1558
Apoptosis							
33	AIFM1	Apoptosis-inducing factor, mitochondrion-associated, 1	NM_001297632	0.96	0.9398	5.71	0.1659
34	AIFM3	Apoptosis-inducing factor, mitochondrion-associated, 3	XM_013983186	1.27	0.6638	0.60	0.4142

35	APAF1	Apoptotic peptidase activating factor 1	XM_003481742	22.85	0.1492	27.95	0.3082
36	BAK1	Bak protein	XM_001928147	0.82	0.6186	0.91	0.8137
37	BAX	BCL2-associated X protein	XM_003127290	16.11	0.1216	48.06	0.2703
38	BCL2	B-cell CLL/lymphoma 2	XM_003121700	5.32	0.0209	2.30	0.08
39	CASP3	Caspase 3, apoptosis-related cysteine peptidase	NM_214131	89.44	0.0889	7.69	0.1063
40	CASP8	Caspase 8, apoptosis-related cysteine peptidase	NM_001031779	2.28	0.292	1.79	0.4851
41	CIDEA	Cell death-inducing DFFA- like effector a	NM_001112696	1.07	0.9031	0.84	0.7327
42	DIABLO	Diablo, IAP-binding mitochondrial protein	NM_001190216	0.49	0.0256	0.55	0.0421
43	DFFA	DNA fragmentation factor subunit alpha-like	XM_003127563	3.11	0.0733	1.76	0.1588
44	FAS	Fas (TNF receptor superfamily, member 6)	NM_213839	14.15	0.0361	0.74	0.531
45	RIPK1	Receptor (TNFRSF)-interacting serine-threonine kinase 1	XM_003128161	1.52	0.6007	0.68	0.4966
46	TNF	Tumor necrosis factor	NM_214022	20.74	0.0091	14.39	0.3567
Growth factors							
47	FGF2	Fibroblast growth factor 2 (basic)	XM_013978917	0.39	0.2403	0.52	0.3647
48	HIF1A	Hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	NM_001123124	9.74	0.0432	2.93	0.42
49	IL10	Interleukin 10	NM_214041	16.16	0.1777	3.85	0.4881
50	MMP2	Matrix metalloproteinase 2 (gelatinase A, 72 kDa gelatinase, 72 kDa type IV collagenase)	NM_214192	0.75	0.5959	1.17	0.7673
51	MMP9	Matrix metalloproteinase 9 (gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase)	NM_001038004	1.01	0.977	0.54	0.1819
52	TIMP1	TIMP metalloproteinase inhibitor 1	NM_213857	1.75	0.3061	1.11	0.8066
53	TGFA	Transforming growth factor, alpha	NM_214251	1.03	0.9594	1.22	0.7434
54	VEGFA	Vascular endothelial growth factor A	NM_214084	7.59	0.0255	1.74	0.1304
55	NGFR	Nerve growth factor receptor	XM_003131565	2.14	0.2902	0.23	0.2853
Macular degeneration and RPE survival							
56	BEST1	Bestrophin 1	XM_005660805	5.11	0.0045	1.37	0.4671
57	CFH	Complement factor H	NM_214281	1.81	0.2513	5.28	0.0263
58	ICAM1	Interacellular adhesion molecule-1	NM_213816	1.31	0.7735	1.20	0.8531
59	NOS1	Nitric oxide synthase 1 (neuronal)	XM_013990334	2.67	0.0905	0.77	0.7234
60	NOS3	Nitric oxide synthase 3 (endothelial cell)	NM_214295	1.61	0.6116	5.55	0.2175
61	RPE65	Retinal pigment epithelium-specific protein 65 kDa	XM_003127931	0.35	0.0177	1.00	0.9974
62	TLR3	Toll-like receptor 3	NM_001097444	3.25	0.0299	0.36	0.0777

63	TLR4	Toll-like receptor 4	NM_001113039	1.87	0.1172	1.56	0.2303
Inflammatory							
64	CCL2=mcp1	Chemokine (C-C motif) ligand 2	NM_214214	0.64	0.3645	1.19	0.7675
65	CXCL2=SDF1	Growth-regulated protein homolog gamma	XM_005652553	0.21	0.1877	0.12	0.1014
66	CXCR4	Chemokine (C-X-C motif) receptor 4	NM_213773	0.18	0.3034	1.35	0.7967
67	IL18	Interleukin 18 (interferon-gamma-inducing factor)	NM_213997	12.41	0.0215	7.29	0.0719
68	IL17A	Interleukin 17 A	NM_001005729	1.96	0.4738	0.44	0.5468
69	IL8	Interleukin 8	NM_213867	6.80	0.0327	1.76	0.2997
70	IL1A	Interleukin 1, alpha	NM_214029	0.76	0.6787	0.06	0.0239
71	IL1B1	Interleukin 1, beta	NM_214055	1.58	0.3767	1.14	0.7981
72	IL6	Interleukin 6 (interferon, beta 2)	NM_214399	5.33	0.0159	9.99	0.0978
73	IL12RB1	Interleukin 12 receptor, beta 1	NM_001145986	5.59	0.0194	2.51	0.0309
74	TLR2	Toll-like receptor 2	NM_213761	2.61	0.2055	0.85	0.8103
75	TLR1	Toll-like receptor 1	NM_001031775	1.65	0.411	2.79	0.4546
Others							
76	ATF4	Activating transcription factor 4 (tax-responsive enhancer element B67)	NM_001123078	4.23	0.2829	0.04	0.2116
77	AQP2	Aquaporine 2 (collecting duct)	NM_001128476	0.57	0.3246	0.32	0.0786
78	AQP3	Aquaporine 3 (Gill blood group)	NM_001110172	0.53	0.1576	0.36	0.0371
79	AQP1	Aquaporine 1 (Colton blood group)	NM_214454	1.10	0.7251	1.50	0.3442
80	EGF	Epidermal growth factor	NM_214020	3.26	0.1327	2.36	0.2133
81	GDNF	Glial cell derived neurotrophic factor	XM_005672424	3.47	0.0608	1.41	0.5638
82	GFAP	Glial fibrillary acidic protein	NM_001244397	2.35	0.1292	0.46	0.1551
83	MMP1	Matrix metalloproteinase 1 (interstitial collagenase)	NM_001166229	0.51	0.1615	1.27	0.7891
84	MMP3	Matrix metalloproteinase 3 (stromelysin 1, progelatinase)	NM_001166308	0.10	0.0076	0.12	0.0044
85	MITF	Microphthalmia-associated transcription factor	NM_001038001	1.99	0.3944	4.88	0.0448
86	PTEN	Phosphatase and tensin homolog	NM_001143696	9.22	0.1284	5.57	0.2605
87	PCNA	Proliferating cell nuclear antigen	NM_001291925	2.85	0.4034	0.56	0.4127
88	SERPINF1	Serpin peptidase inhibitor, clade F (alpha-2 antipain, pigment epithelium derived factor), member 1	NM_001078662	1.06	0.8347	0.64	0.1454
89	SERPINE1	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	NM_213910	5.93	0.1141	2.69	0.3189
90	NFE2L2	Nuclear factor (erythroid-derived 2)-like2	XM_003133500	1.84	0.4031	1.43	0.5989
91	TIMP3	Tissue inhibitor of metalloproteinase- 3	XM_003126073	2.53	0.3303	0.95	0.9642

Drop in the focal ERG signal amplitude precedes lesion generation

Fig. S3 shows the amplitude change of the fERG responses for 5 mm spots collected during laser exposure at peak temperature and the recovery of the amplitude after the retina returns to normal body temperature. The amplitude did not change drastically at 44 °C but dropped precipitously when the temperature was increased further. After termination of non-damaging laser exposures, the signal amplitude typically recovered near pre-treatment levels. However, in treatments producing a lesion, the fERG signal amplitude first reduced to 32 ± 11 % (mean $\pm$ std) but recovered only to 67 ± 8.8 % (mean $\pm$ std) after the treated area had returned to body temperature.

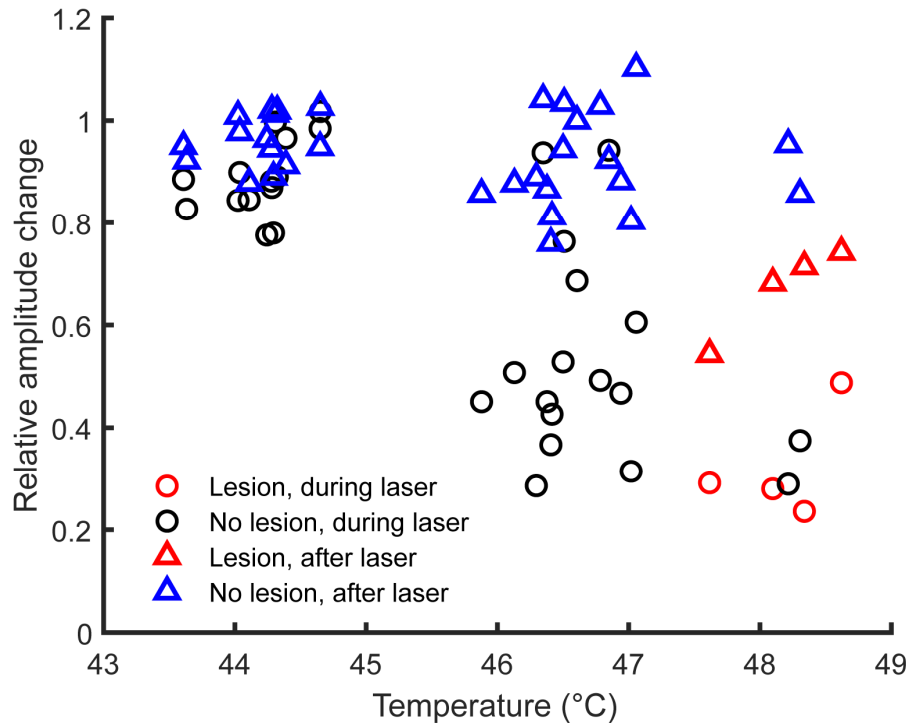


Figure S3. Effects of retinal hyperthermia on fERG response amplitude

Relative changes in fERG amplitudes during (black circles) and after the laser exposure (blue triangles) compared to fERG amplitudes recorded before the laser exposure. Treatments that caused lesions are highlighted with red. fERG amplitudes during the treatment are acquired 30 to 60 seconds after the initiation of laser exposure while the responses after the laser exposure are collected from 30 to 60 seconds succeeding the laser turn-off. The x-axis shows the steady-state temperature of each laser exposure determined with the ERG-based laser power calibration protocol. Source data are provided as a Supplementary Information Source Data file.

Focal ERG-stimulation for temperature determination

Obtaining ERG-based local temperature information from the heated retinal area requires focal ERG recording. The optimal fERG stimulation for temperature determination should elicit high amplitude responses with a fast signal repetition rate to maximize the signal-to-noise ratio and temporal resolution of the method. Fig. S1 A shows exemplary fERG impulse responses recorded with a 4 mm diameter stimulus spot using the uneven flicker protocol (see Methods). The three responses were elicited with flash stimuli with luminous exposures of 23, 46, and 92 lx·s against a constant white

background with 1150 lx illuminance. The white background was delivered in an 8 mm diameter circular spot, which also enabled visualization of the highly pigmented pig fundus during the experiments. Additionally, a peripheral background light of 2300 lx, was projected to the fundus to minimize the effect of stray light to the fERG signal (see Fig. 1 B for optical configuration). A luminous exposure of 46 lx·s was selected for fERG stimulation during laser treatments because it elicits high amplitude non-saturated fERG responses with the used background illumination. The fERG response amplitude per stimulation area, measured from the peak of the a-wave to the peak of the b-wave, was 220 ± 73 nV/mm² (mean $\pm$ std, n = 72) with the chosen stimulus strength at normal body temperature.

Occasionally, persistent oscillating waveforms tailed the b-wave of the fERG signal and overlapped with the a- and b-waves generated by the subsequent flash. Typically, the effect of signal overlap was mild but, in rare cases, oscillations significantly affected the early fERG waveforms elicited with a steady 16 Hz flickering stimulus. An uneven flicker protocol was deployed instead of a steady flicker to reduce the effect of the oscillations after the experiments with 6 pigs (see detailed description in Methods). Fig. S1 B shows fERG responses from the same 2.7 mm diameter retinal area elicited with a steady 16Hz flicker and with the uneven flicker protocol. The effect of the signal overlap is visible mainly in the early part of the blue trace recorded with steady flicker but disappears when using the uneven flicker protocol (orange trace). Results obtained with uneven flicker, as well as results with steady flicker, where the effect of signal overlap was mild, were not separated in further analysis. However, results obtained with the constant frequency flicker with extremely strong signal overlap were disregarded. Source data are provided as a Source Data file.

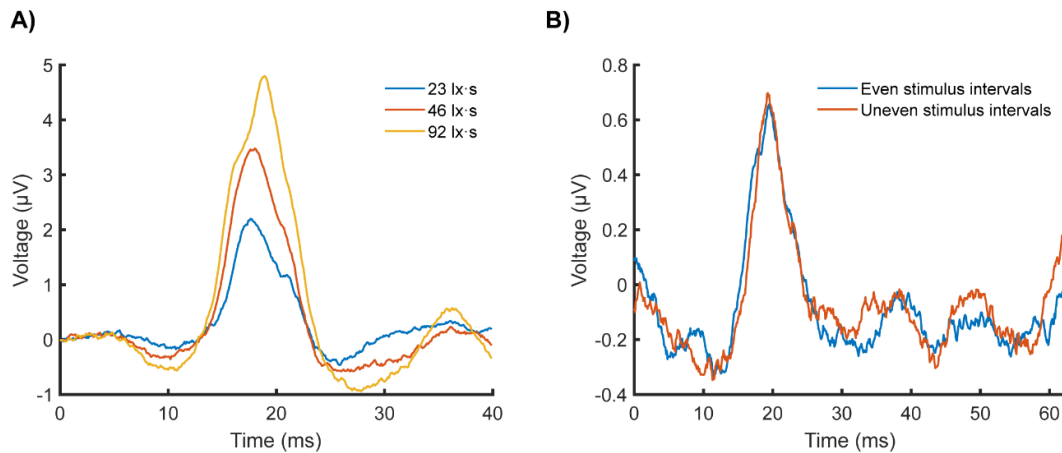


Figure S4. Focal ERG from pig cone photoreceptors

A) fERG responses acquired with three flash strengths. **B)** Comparison of the 16 Hz flicker stimulation (blue trace) with uneven flicker protocol (red trace). Signal distortion due to overlapping signals is visible in the early negative wave collected with steady 16 Hz flicker but does not appear in the trace collected using the uneven flicker protocol. Source data are provided as a Supplementary Information Source Data file.

Protocols for ERG-based laser power calibration

The local thermal responsivity was determined before each laser treatment by using two different calibration protocols. The first protocol was used for treatments with 3.4 mm laser spot and 2.7 mm

fERG stimulation spot diameters. In the protocol, the retinal temperature was elevated with three separate 60-second laser exposures, allowing the retinal temperature to return to body temperature before the subsequent laser exposure (Fig. S5A). The first 15 s of the heating were reserved for temperature stabilization and the fERG responses collected during the following 45 seconds of laser exposure were used for estimating the local thermal responsivity. The fERG responses collected during the exposure were compared to pre-exposure fERG, recorded for 45 seconds directly preceding the laser heating.

In the second calibration protocol, the laser spot diameter was increased to 5 mm and fERG stimulation to 4 mm. Owing to a higher signal-to-noise ratio of fERG signal with a larger spot size, we could shorten the calibration procedure to three consecutive 30-second exposures, conducted with different powers without turning the laser off in between (Fig. S5B), preceded by the recording of 15-second reference fERG response. The first 15 s of each laser exposure were reserved for temperature stabilization and the last 15 s were used for temperature determination. The temperature elevations caused by the calibration laser exposures were calculated from the acceleration of the ERG responses as described in the Methods.

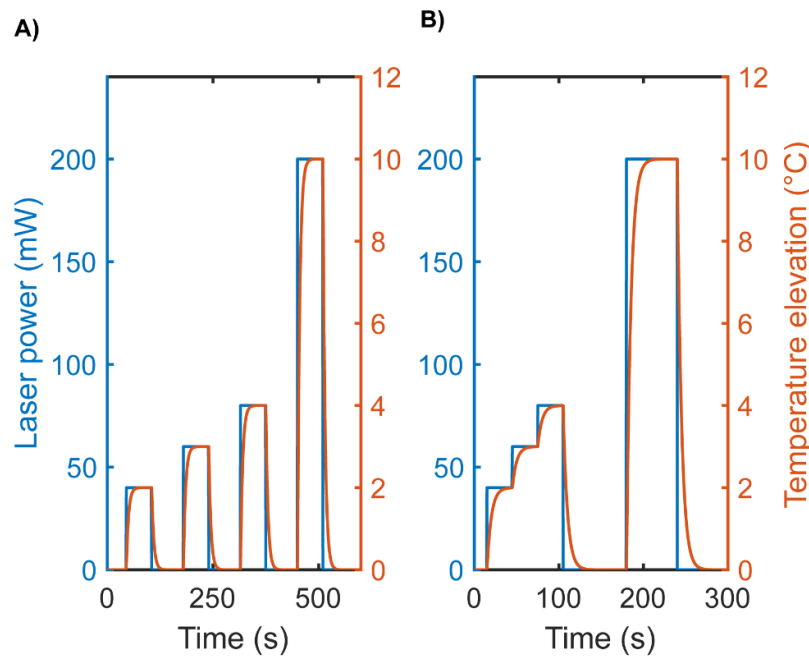


Figure S5. Illustration of the individualized laser power calibration protocols.

The blue traces illustrate laser powers and the red traces show indicative retinal temperature elevations during the two alternative ERG-based laser power calibration protocols used in the study. A) The first protocol comprises three separate 60-second laser exposures, allowing retinal temperature to normalize in between. B) The second protocol comprises three consecutive 30-second laser exposures. The calibration protocols are followed by 60-second laser treatment.

Table S2. Protocols used for determination of therapeutic temperature window and quantitative analysis of heat-induced biochemical effects.

	Determination of therapeutic temperature window	Quantitative analysis of heat-induced biochemical effects
Laser / ERG stimulation spot diameter (mm)	3.4 / 2.7	5.0 / 4.0
N pigs/eyes/spots	7 / 10 / 36	4 / 8 / 36
Thermal calibration protocols	Three separate 60-second laser exposures	Three consecutive 30-seconds laser exposures
Treatment length (s)	60	60
ERG stimulation protocols	16Hz flicker (26 spots) and uneven flicker (10 spots)	Uneven flicker
Analysis after enucleation	Whole flat mount immunostaining	qPCR and Immuno blotting

Statistical analysis of lesion and HSP70 threshold temperatures

Probit model

The retinal temperature of each of the treatments, T_n , was estimated with the ERG-based laser power calibration protocol. The temperature estimate $T_{ERG,n}$ is expected to contain a normally distributed error with standard deviation σ_T .

$$T_n = T_{ERG,n} + N(0, \sigma_T) \quad (S1)$$

The formation of a visible lesion was modeled as a binary event that occurs above a certain threshold temperature for the treated area $T_{lesion,n}$, which is expected to be normally distributed around the ED50 temperature for lesion generation.

$$T_{lesion,n} = ED50_{lesion} + N(0, \sigma_{lesion}) \quad (S2)$$

Each treatment was labeled as either causing or not causing a lesion ($L_n = 1$ for lesion, $L_n = 0$ for no lesion). Hence, the probability for a given treatment to cause a lesion, shown in Eq. S3, corresponds to the likelihood that the actual retinal temperature was above the lesion threshold temperature for the treated area.

$$P(L_n = 1) = P(T_n > T_{lesion,n}) = T_{ERG,n} + N(0, \sigma_T) > ED50_{lesion} + N(0, \sigma_{lesion}) \quad (S3)$$

The error terms for retinal temperature determination and possible deviations in the lesion threshold temperature are combined into a single error term $\sigma_{total} = \sqrt{\sigma_T^2 + \sigma_{lesion}^2}$. The probability of generating a lesion is shown in Eq. S4 using the cumulative normal distribution Φ with mean $ED50_{lesion}$ and standard deviation σ_{total} .

$$P(L_n = 1) = P(T_{ERG,n} > ED50_{lesion} + N(0, \sigma_{total})) = 1 - \Phi_{T_{lesion}, \sigma_{total}}(T_{ERG,n}) \quad (S4)$$

Using a flat prior, the posterior distribution of $ED50_{lesion}$ and σ_{total} is the same as the likelihood of the observed data.

$$L(ED50_{lesion}, \sigma) = \prod_{n=1}^N \left(1 - \Phi_{ED50_{lesion}, \sigma_{total}}(T_{ERG,n})\right)^{L_n} \left(\Phi_{ED50_{lesion}, \sigma_{total}}(T_{ERG,n})\right)^{L_n - 1} \quad (S5)$$

The reported ED50 temperatures for lesion generation and the standard error in the determined retinal temperature are the values that maximize this likelihood function. The σ_{total} can be seen as an upper bound for the error in retinal temperature determination, as it contains deviations in the lesion threshold temperature as well as the temperature determination error. The reported 95% confidence intervals refer to the intervals of $ED50_{Lesion}$ and σ_{total} that correspond to 95% of total likelihood.