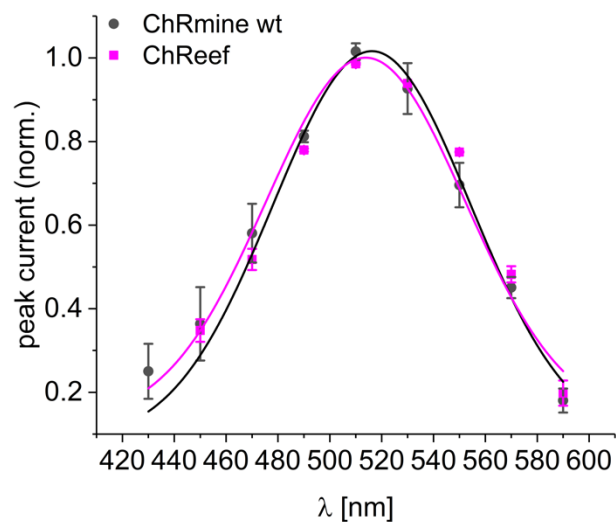


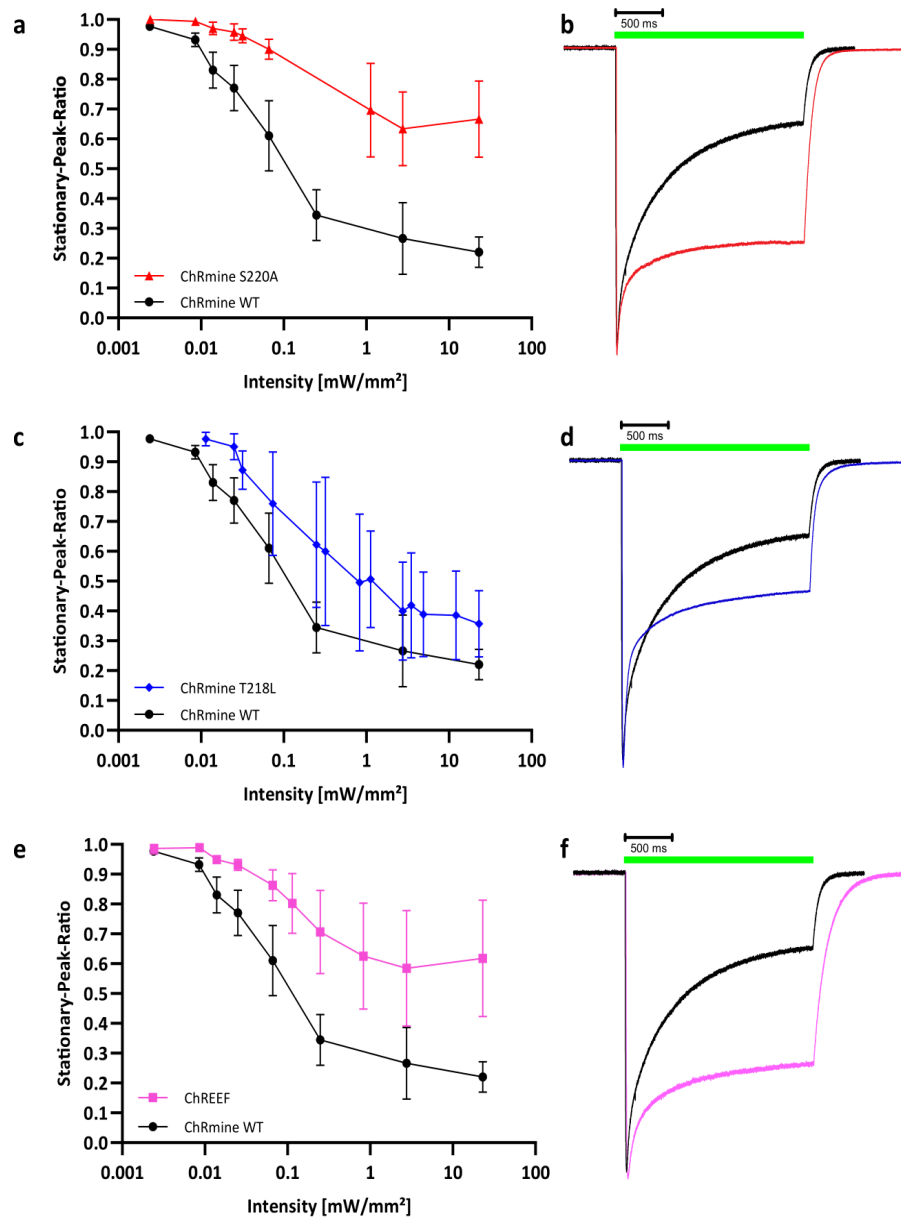
Supplementary Material

List of supplementary material:

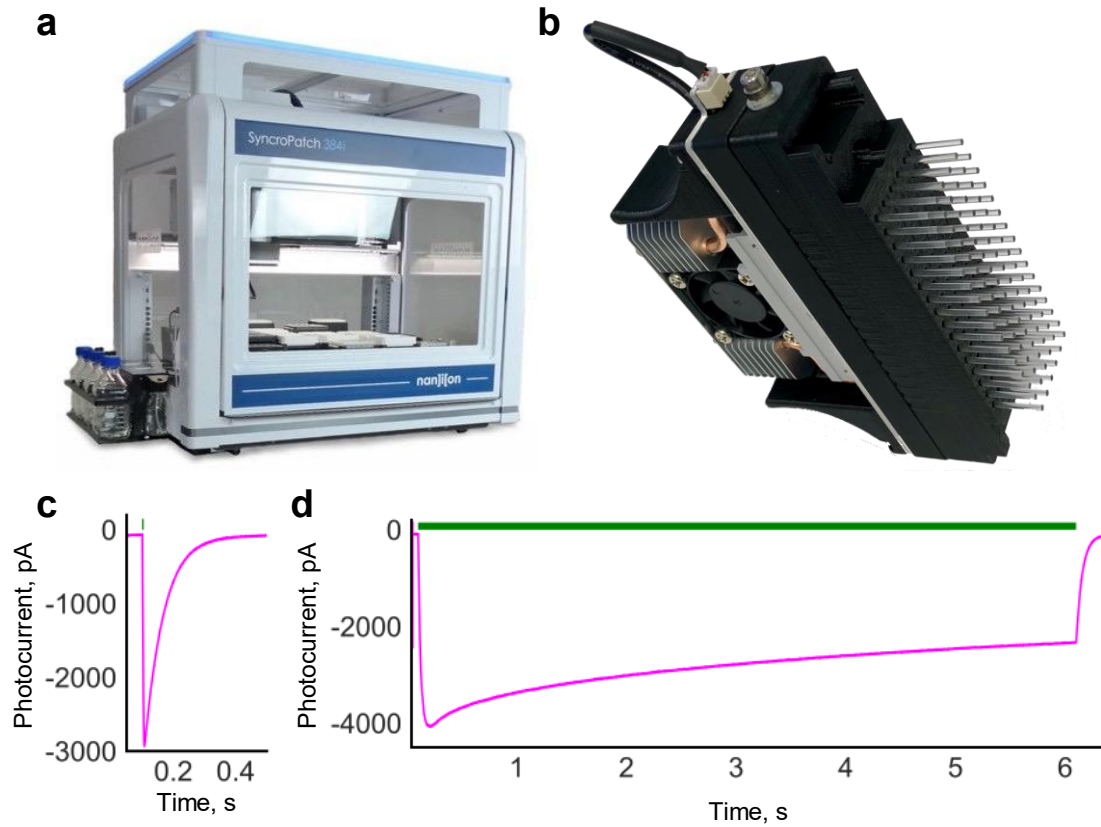
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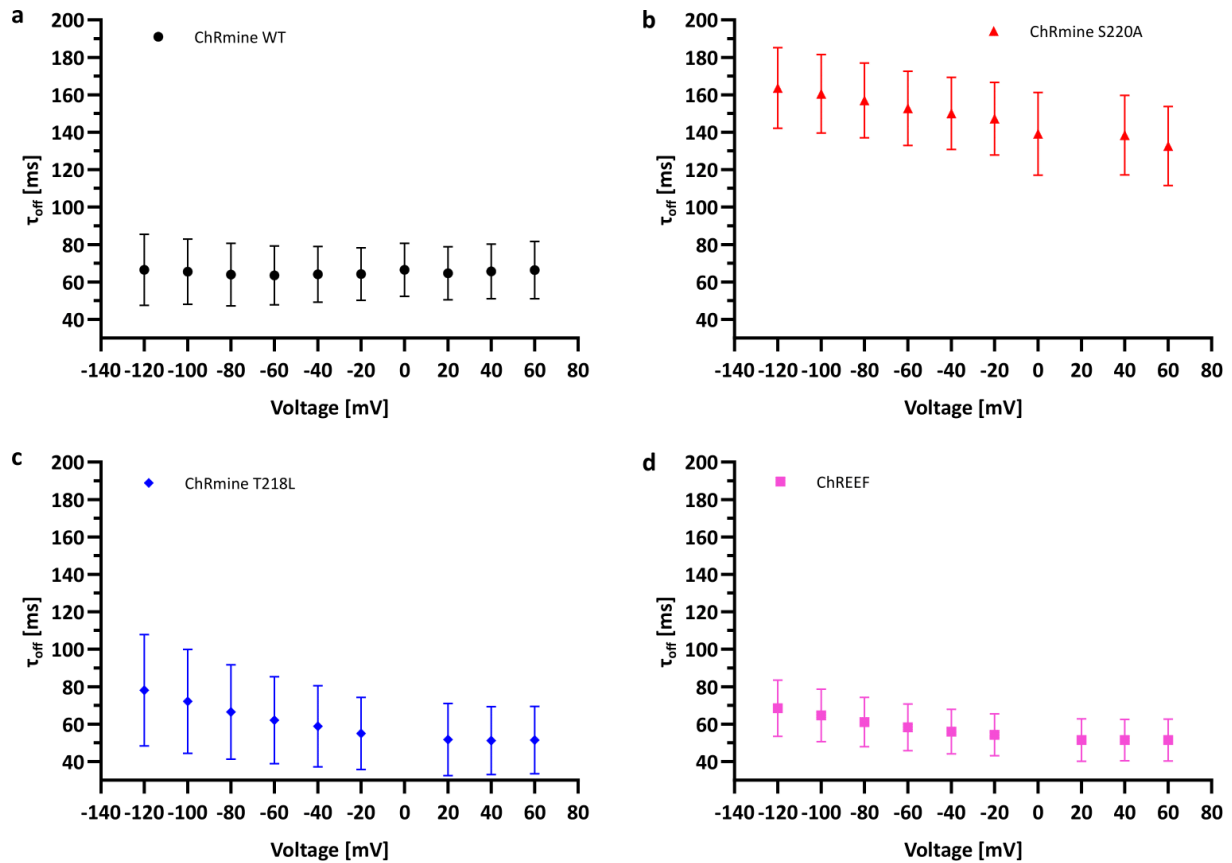
Supplementary Figure 1: Action spectra of ChRmine wt (black circles, n=3) and ChReef (magenta squares, n=3). Shown are normed peak currents in response to ns light-pulses of the indicated wavelengths measured in whole-cell patch-clamp experiments at a membrane potential of -60 mV. The pulse energies at the different wavelengths were set to equal photon counts. The symbols indicate average values, the error bars show SD.



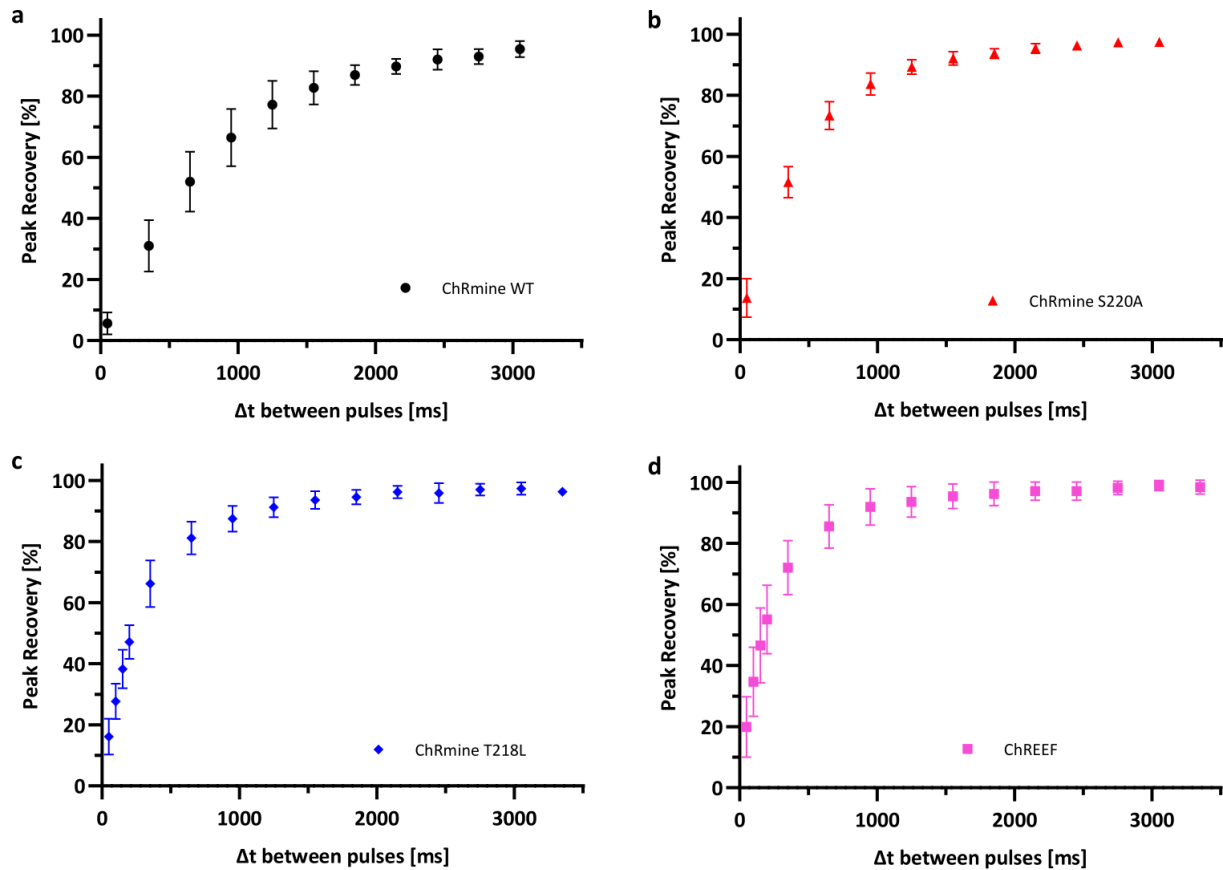
Supplementary Figure 2: Desensitization of ChRmine variants at different light intensities. NG cells transiently transfected with ChRmine (black circle, WT), ChRmine S220A (red triangle), ChRmine T218L (blue rhombus) and ChReef (magenta squares) were investigated by whole-cell patch-clamp recordings at a membrane potential of -60 mV. Photocurrents were measured upon illumination with a 2 s light pulses of a wavelength of $\lambda=532$ nm at different light intensities ranging from 0.0024 mW/mm² to 23 mW/mm². The stationary-peak-ratios were calculated as the quotient of the mean stationary current of the last 100 ms of the 2s light pulse and the peak current. Shown are the light dependencies of the stationary-peak-ratios for **(a)** ChRmine S220A (n=4), **(c)** ChRmine T218L (n=4) and **(e)** ChReef (n=4) in comparison to ChRmine (n=5). Exemplary photocurrent traces at saturating light intensity (23 mW/mm²) are depicted for **(b)** ChRmine S220A (red), **(d)** ChRmine T218L (blue) and **(f)** ChReef (magenta) in comparison to ChRmine (black). Error bars show SD.



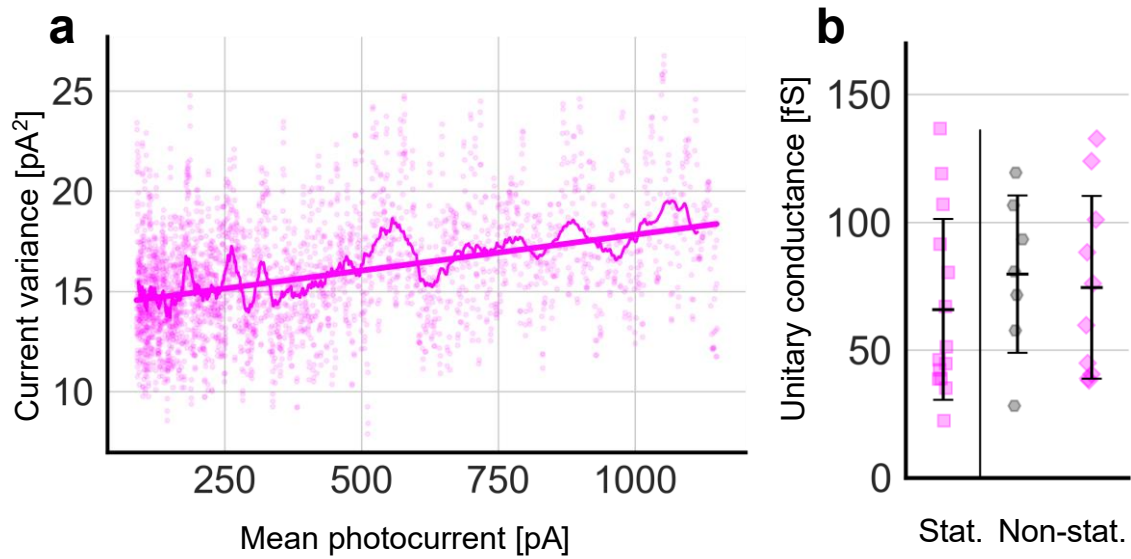
Supplementary Figure 3. Automated photocurrent measurements. **a**, Picture showing the high performance automated patch-clamp system SyncroPatch384 (Nanion) **b**, Picture showing the illumination unit (Nanion), which comprises 96 LEDs coupled to 96 individual light fibers and a cooling system with a fan. **c**, Exemplary ChReef photocurrent at a membrane potential of -100 mV evoked by a 5-ms green light pulse, which was used for non-stationary noise analysis. **d**, Exemplary ChReef photocurrent at a membrane potential of -100 mV evoked by a 6-s green light pulse, which was used for stationary noise analysis.



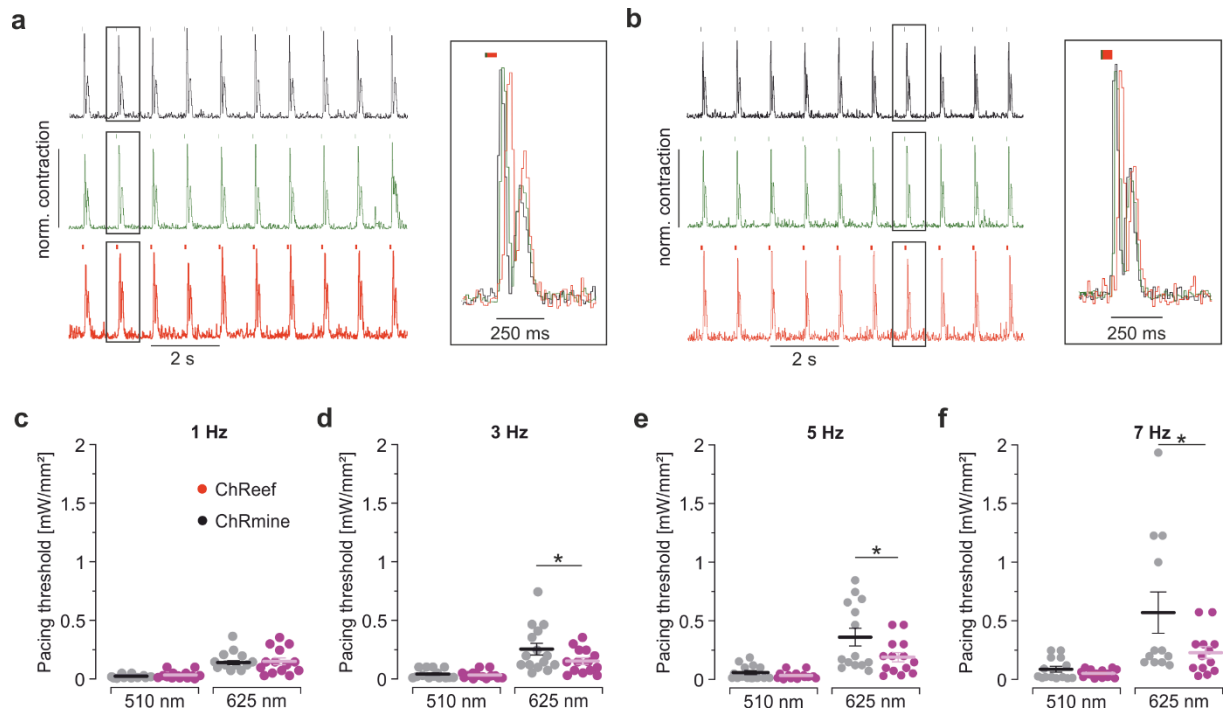
Supplementary Figure 4: Voltage dependence of closing kinetics of ChRmine variants. NG cells transiently transfected with ChRmine (black circle, WT), ChRmine S220A (red triangle), ChRmine T218L (blue rhombus) and ChRmine S220A (magenta square) were investigated by whole-cell patch-clamp recordings at different membrane potentials ranging from -120 mV to 60 mV. Photocurrents were measured upon illumination with a 3 ms light pulse of a wavelength of $\lambda=532$ nm at a light intensity of 23 mW/mm². The closing kinetics (τ_{off} value) were determined by a fit of the decaying photocurrent elicited in response to the light pulse to a monoexponential function. Shown are the time constants of the closing kinetics τ_{off} for **(a)** ChRmine WT (n=7), **(b)** ChRmine S220A (n=6), **(c)** ChRmine T218L (n=7) and **(d)** ChRmine S220A (n=7). The symbols indicate the mean, error bars show SD.



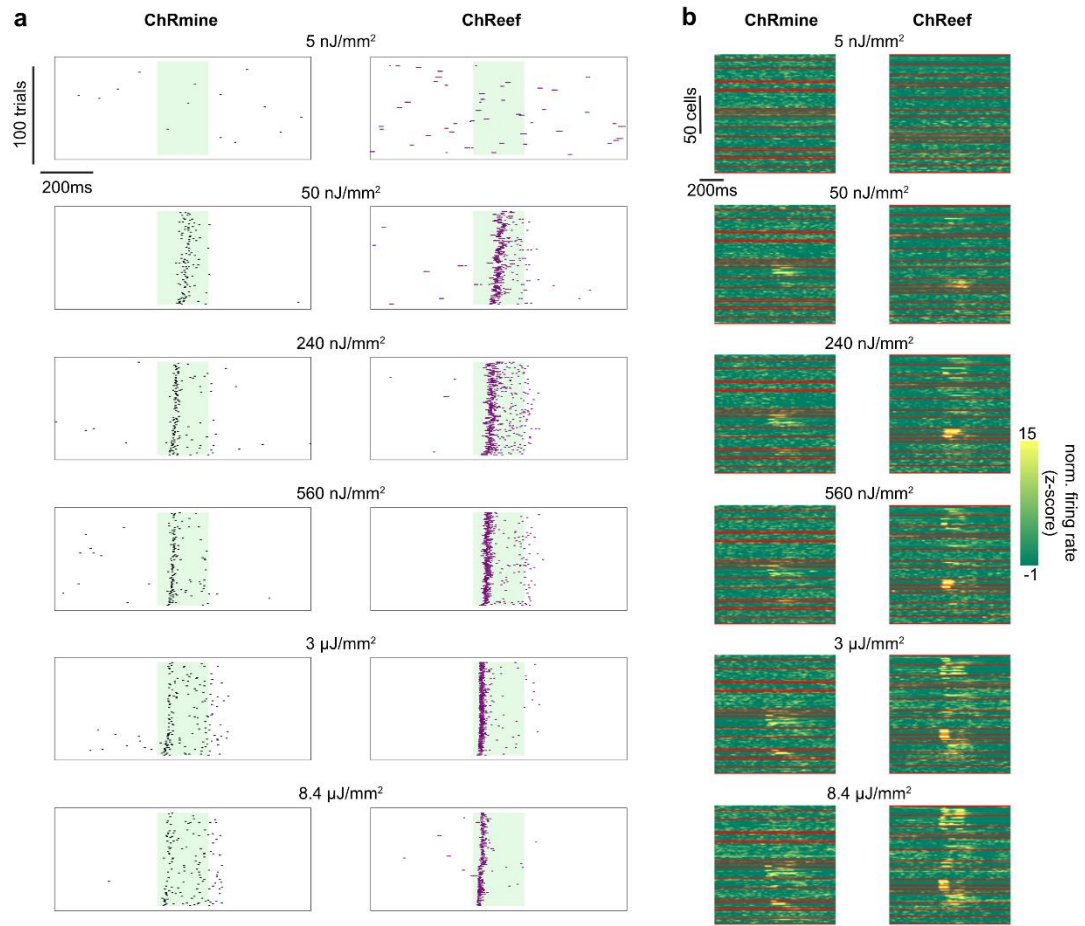
Supplementary Figure 5: Peak recovery of ChRmine variants. NG cells transiently transfected with **(a)** ChRmine WT (black circle, $n=4$), **(b)** ChRmine S220A (red triangle, $n=4$), **(c)** ChRmine T218L (blue rhombus, $n=5$) and **(d)** ChRmine S220A (magenta square, $n=5$) were investigated by whole-cell patch-clamp recordings at a membrane potential of -60 mV. Photocurrents were measured upon illumination with two subsequent 3 s light pulses of a wavelength of $\lambda=532$ nm at a light intensity of 23 mW/mm² with a varying time between the light pulses (Δt between pulses, ms). Peak recovery was calculated as the quotient of the difference between the peak and stationary current of pulse two ($I_{p2}-I_{s2}$) and peak and stationary current of pulse one ($I_{p1}-I_{s1}$) in %. The symbols indicate the mean, error bars show SD.



Supplementary Figure 6. Noise analysis of ChReef and ChRmine photocurrents **a**, Non-stationary noise analysis of ChReef. Exemplary plot showing the relation between the variance of the photocurrent and its average value. The photocurrents were measured in whole-cell patch-clamp experiments at a membrane potential of -60 mV. The thin line shows the moving average of the variance. The bold line indicates a linear fit. **b**, Statistical comparison of single channel conductance values at a membrane potential of -60 mV. The single channel conductance values of ChReef (magenta diamonds) and ChRmine (grey hexagons) derived from non-stationary noise analysis are shown on the right. On the left side the single channel conductance value of ChReef (magenta squares) derived from stationary noise analysis is shown.

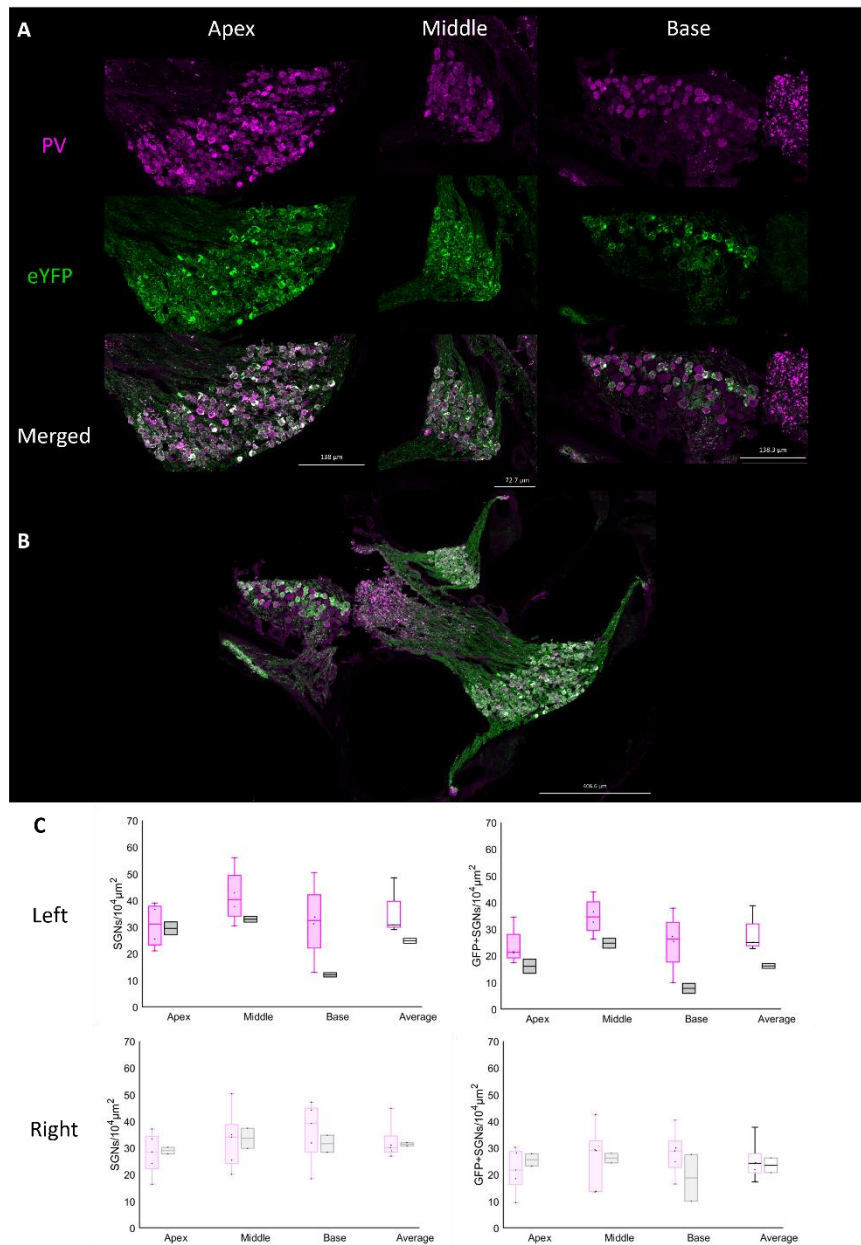


Supplementary Figure 7. Optical pacing of cardiac cell clusters expressing ChRmine and ChReef. **a**, Representative traces of normalized contractility of a cardiac cell cluster expressing ChRmine upon electrical stimulation (top: black lines, 0.2 ms biphasic, 45 V), green light (middle: green lines, 510 nm, 5 ms, 50 $\mu\text{W}/\text{mm}^2$) and red light (bottom: red lines, 625 nm, 50 ms, 250 $\mu\text{W}/\text{mm}^2$). **b**, Representative traces of normalized contractions of a ChReef expressing cardiac cell cluster upon electrical stimulation (top: black lines, 0.2 ms biphasic, 45 V), green light (middle: green lines, 510 nm, 5 ms, 50 $\mu\text{W}/\text{mm}^2$) and far-red light (bottom: red lines, 625 nm, 50 ms, 150 $\mu\text{W}/\text{mm}^2$). **c-f**, Aggregated data of the pacing threshold using green and red light for ChRmine (black) and ChReef (red) for increasing pacing rates: 1 Hz (**c**, $p(510 \text{ nm}) = 0.86$; $p(625 \text{ nm}) = 0.90$), 3 Hz (**d**, $p(510 \text{ nm}) = 0.98$; $p(625 \text{ nm}) = 0.043$), 5 Hz (**e**, $p(510 \text{ nm}) = 0.90$; $p(625 \text{ nm}) = 0.016$) and 7 Hz (**f**, $p(510 \text{ nm}) = 0.95$; $p(625 \text{ nm}) = 0.018$). Statistical analysis was performed using a 2way ANOVA with Sidaks multiple comparison test ($N = 2$ independent isolations and AAV transductions; $n \geq 12$ individual cardiac cell clusters indicated by single dots).

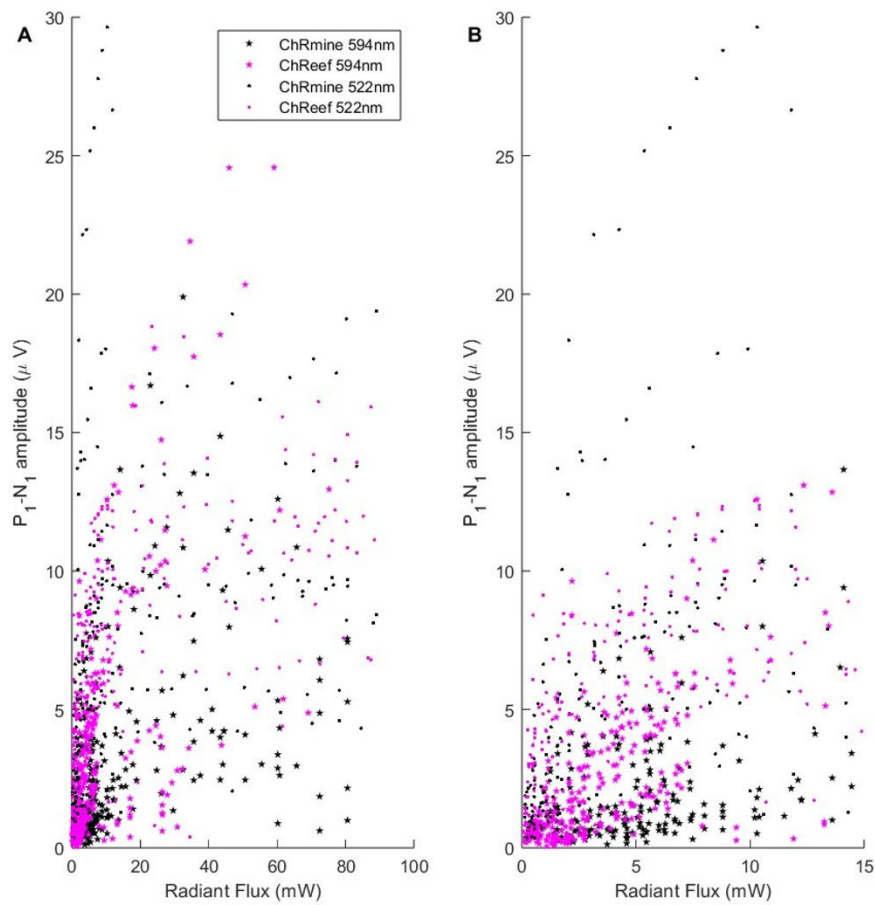


Supplementary Figure 8. ChReef-mediated vision restoration in rd1 mice is reliable with low light requirements.

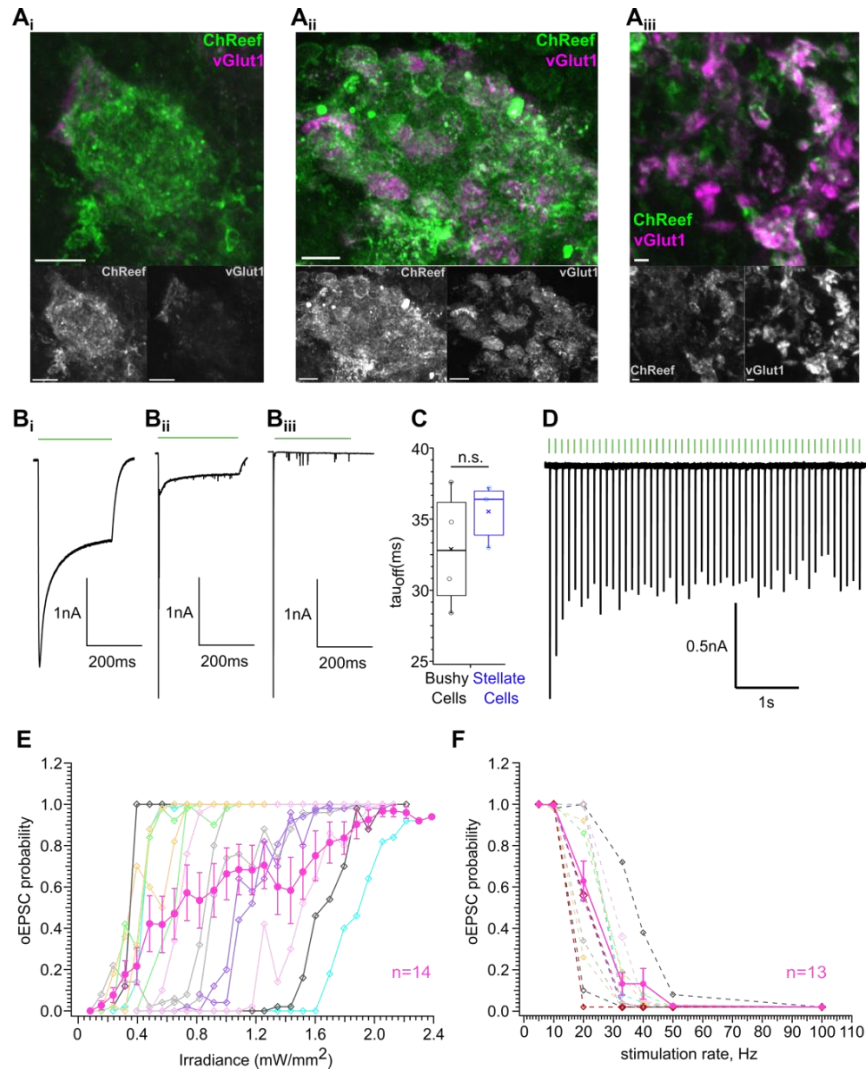
a, Raster plots of two highly sensitive, example single units recorded in V1 of ChRmine- and ChReef-injected mice in response to various light intensities as indicated. In such highly sensitive cells, we observed robust responses at light intensities as low as 0.25 μW/mm² (50 nJ/mm²). **b**, All recorded cells of both conditions and their responses to the same light intensities as in a. Red lines separate cells from different recording sites.



Supplementary Figure 9. Immunohistochemical analysis of ChReef expression in the AAV-injected mouse cochlea. **a**, Exemplary left cochlea of mouse injected with AAV2/9-ChReef imaged at 40X across all cochlear turns (staining: anti-parvalbumin as context marker, anti-GFP detecting ChR-EYFP, and merged shown from top to bottom respectively). **b**, Overview scan imaged at 20X of exemplary cochlea show robust expression of ChReef in SGNs of the apex, middle and basal turns of the cochlea. **c**, Box plots showing SGN density and density of GFP positive cells across all cochlear turns in both the left and right cochleae for ChReef (magenta) and ChRmine (black) expression mice.

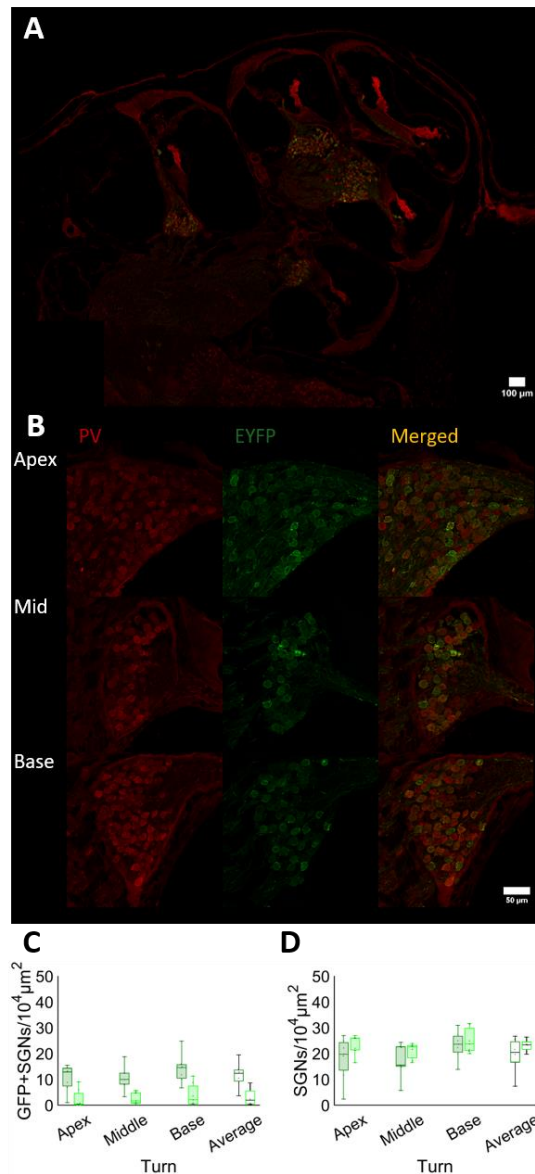


Supplementary Figure 10. oABR P1-N1 amplitudes mediated by ChReef increase with radiant flux. Acquired from oABR measurements in mice expressing ChReef (magenta) or ChRmine (black) using green (circle) or orange (star) light stimulation. **a**, Full spectrum of all radiant flux values, and **(B)** Visualization of the radiant flux values most densely sampled (~0 to 15 mW).



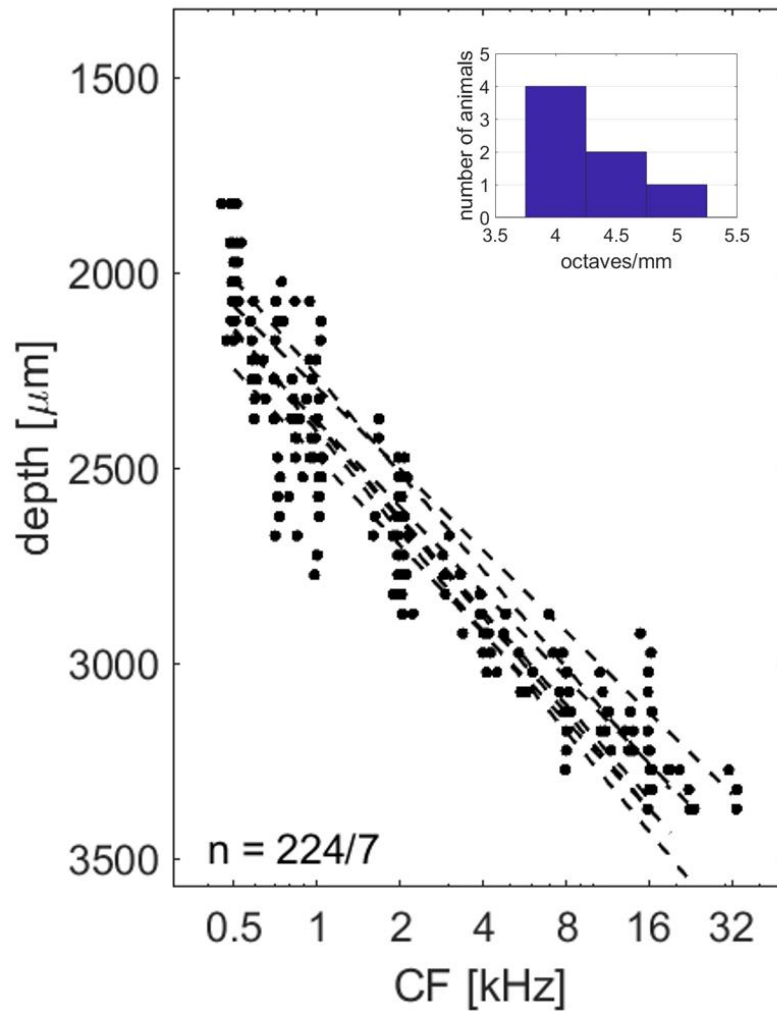
Supplementary Figure 11. Utility of ChReef in brain slice physiology.

a_i - a_{iii}, Parasagittal AVCN slices were harvested from AAV-injected mice at p20-p23 and fixed for 30 minutes in formaldehyde. The slices were immunolabelled against vGLUT1 and GFP to track the expression of ChReef across the SGN terminals (**a_i**) and/or transduced postsynaptic AVCN neurons (**a_{ii}/a_{iii}**). **b_i - b_{iii}**, Voltage clamp recordings from BCs at near-physiological temperature held at -70 mV. Exposure to green light triggers ChReef photocurrents in directly transduced BCs (**b_i**), an overlay of EPSCs and direct currents, when both the endbulb of Held and the BC express ChReef (**b_{ii}**) and optically evoked EPSCs (oEPSCs), when only the endbulb expresses the channelrhodopsin (**b_{iii}**). **c**, The closing kinetics of ChReef were measured during voltage clamp recordings from Bushy and stellate cells. The exponential decay component's τ_{off} was not significantly different between the two cell types. **d**, Train of 50 oEPSCs resulting from 1 ms light pulses delivered at 10 Hz. **e**, Different laser intensities were tested with 50 stimuli trains delivered at 10 Hz to determine the oEPSC probability as $n_{\text{successful oEPSCs}}/50$. Spike probability is plotted against the tested irradiance values for each voltage clamped BC (multi-colored traces). The average spike probability across all BCs for each irradiance value is depicted as the dark magenta trace and overlaid on the graph. **f**, BCs were exposed to 1 ms light pulses at different frequencies, using the smallest irradiance value that yielded an oEPSC probability of 1 at 10 Hz. The oEPSC probability across different frequencies is depicted for each BC included in the analysis (multi-colored traces). The average oEPSC probability across BCs for each frequency is overlaid as the dark magenta trace. Bars indicate SD.

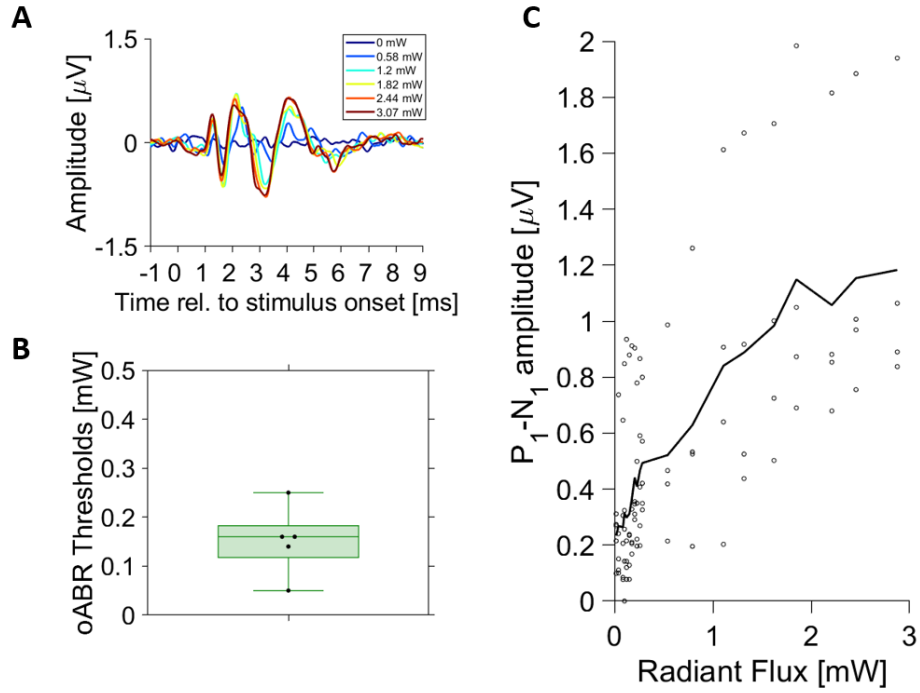


Supplementary Figure 12. Immunohistochemical analysis of ChReef expression in the AAV-injected gerbil cochlea.

a, Exemplary left cochlea overview imaged at 20X displays expression across all turns **b**, Exemplary left cochlea imaged at 40X shows ChR expression in spiral ganglion neuron (SGN) across all cochlear turns (staining: anti-parvalbumin as context marker, anti-GFP detecting ChR-EYFP and merged shown from left to right resp.) **c**, Boxplot computing GFP positive SGN cells as a density function of ChR-expressing SGN cells (GFP+) to the area of Rosenthal's canal observed on slice. (dark green: injected left cochleae, light green: right non-injected cochleae). Cell counting is performed using a custom MATLAB tool that performs assisted automatic selection of SGN. Neurons are delineated and ChR expressing cells contrasted to non-expressing cells through principal component analysis. **d**, Boxplot computing SGN as a density function of SGN cells to the area of Rosenthal's canal observed on slice. (dark green: injected left cochleae, light green: right non-injected cochleae).



Supplementary Figure 13. Inferior colliculus recordings: tonotopy. Characteristic frequencies (CF) as a function of recording depth. Dashed line: linear fit of all CFs for each animal, according to Pearson's correlation coefficient. Inset displays distribution of tonotopic slopes computed from linear fits across all animals.



Supplementary Figure 14. Optically evoked ABR in Mongolian gerbils expressing ChReef. a, Exemplary oABR trace. **b,** oABR thresholds computed across animals included ($n=5$). Animals included had a clear, readable and artifact-free oABR trace. **c,** First peak amplitude measured and computed across animal. Average is plotted in bold line, individual datapoints computed in dark circle.

Supplementary Table 1. Stationary-Peak-Ratio, EC₅₀ values, stationary current densities and τ_{off} values of ChRmine variants.

ChR variant	Stationary-Peak-Ratio	EC ₅₀ [$\mu\text{W}/\text{mm}^2$]	J _{-60 mV} [pA/pF]	τ_{off} [ms]
ChReef (a)	0.62 $\pm$ 0.15 # b****,c**	20 $\pm$ 8	97.6 $\pm$ 65.0 \$ b****,c*,d***	58.3 $\pm$ 12.5 § d****
ChRmine (b)	0.22 $\pm$ 0.12 # a****,c****,d****	14 $\pm$ 5	21.6 $\pm$ 15.8 \$ a****,c****,d***	63.5 $\pm$ 15.7 § d****
ChRmine T218L (c)	0.44 $\pm$ 0.13 # a**,b****,d****	29 $\pm$ 15	64.8 $\pm$ 38.8 \$ a*,b****	59.1 $\pm$ 21.3 § d****
ChRmine S220A (d)	0.62 $\pm$ 0.14 # b****,c****	31 $\pm$ 17	54.3 $\pm$ 27.2 \$ a***,b****	152.7 $\pm$ 9.8 § a****,b****,c****

NG cells, transiently transfected with the shown ChRmine variants were investigated by whole-cell patch-clamp recordings at a membrane potential of -60 mV. Photocurrents were measured upon illumination with green light pulses (saturating intensity of 23 mW/mm², λ =532 nm) of 2 s for stationary current density determination and of 3 ms for τ_{off} determination. The stationary current densities (J_{-60mV}) were calculated as the quotient of the mean stationary current and the capacitance of the cell. The τ_{off} values were determined by a fit of the decaying photocurrent, immediately after cessation of the light pulse, to a monoexponential function. The half maximal activation (EC₅₀) was determined by hyperbolic fitting. For ChRmine wt the fitting range was restricted to the range, which could be approximated by a hyperbolic function. The stationary-peak-ratio was calculated as the quotient of the mean stationary current of the last 100 ms of the 2s light pulse and the peak current. # Significantly different Stationary-Peak-Ratio compared to **(a)** ChReef **(b)** ChRmine **(c)** ChRmine T218L **(d)** ChRmine S220A. The EC₅₀ values of ChReef (n=4), ChRmine (n=6), ChRmine T218L and ChRmine S220A (n=4) are not significantly different \$ Significantly different current density compared to **(a)** ChReef **(b)** ChRmine **(c)** ChRmine T218L **(d)** ChRmine S220A § Significantly different τ_{off} compared to **(a)** ChReef **(b)** ChRmine **(c)** ChRmine T218L **(d)** ChRmine S220A. (ANOVA and Bonferroni post hoc test: * p < 0.05 ** p < 10⁻² *** p < 10⁻³ **** p < 10⁻⁴). Shown are mean and standard deviation (SD). The stationary-peak-ratios, stationary current densities and τ_{off} values employed for the statistical comparisons are shown in Figure 1.

Supplementary Table 2. Stationary current densities and τ_{off} values of ChR variants.

ChR variant	$J_{-60 \text{ mV}}$ [pA/pF]	τ_{off} [ms]
ChReef (a)	97.6 ± 65.0 # b***,c***,d****,e****,f***	58.3 ± 12.5 § b****,c****,d****,
Catch (b)	37.0 ± 12.5 # a***	34.4 ± 7.3 § a****,d****,f****
ChR2 T159C (c)	30.6 ± 18.4 # a***	28.3 ± 7.4 § a****,d****,f****
Chronos (d)	24.0 ± 7.5 # a****	3.5 ± 0.6 § a****,b****,c****,e****
Chrimson (e)	24.0 ± 6.8 # a****	23.6 ± 1.5 § a****,d***,f**,
f-Chrimson (f)	34.2 ± 12.7 # a***	5.7 ± 0.4 § a****,b****,c****,e**

NG cells transfected with the shown ChR variants were investigated by whole-cell patch-clamp recordings at a membrane potential of -60 mV. Photocurrents were measured upon illumination with saturating light pulses of 0.5 s or 2 s for stationary current density determination and of 3 ms for τ_{off} determination. The stationary current densities ($J_{-60 \text{ mV}}$) were calculated as the quotient of the mean stationary current and the capacitance of the cell. The τ_{off} values were determined by a fit of the decaying photocurrent, immediately after cessation of the light pulse, to a monoexponential function. # Significantly different current density compared to **(a)** ChReef **(b)** Catch **(c)** ChR2 T159C **(d)** Chronos **(e)** Chrimson **(f)** f-Chrimson § Significantly different τ_{off} compared to **(a)** ChReef **(b)** Catch **(c)** ChR2 T159C **(d)** Chronos **(e)** Chrimson **(f)** f-Chrimson. (ANOVA and Bonferroni post hoc test: * $p < 0.05$ ** $p < 10^{-2}$ *** $p < 10^{-3}$ **** $p < 10^{-4}$). Shown are mean and standard deviation (SD). The stationary current densities and τ_{off} values employed for the statistical comparisons are shown in Figure 1.

Supplementary Table 3. List of primers used for ChRmine mutant generation.

	ChRmine T218L	ChRmine S220A	ChRmine T218L/S220A
template	humanized ChRmine	humanized ChRmine	humanized ChRmine T218L
sequence of forward primer	5'-GTTGCGCCGTG TTTCTGTTCTCCA TGCTGTG-3'	5'-GTGTTTACCTT CGCCATGCTG TGGATTC-3'	5'-CGTGTTTCTG TTCGCCATGCT GTGGATTCTG -3'
sequence of reverse primer	5'-CACAGCATGG AGAACAGAAACAC GGCGAAC-3'	5'- GAATCCACAG CATGGCGAAG GTAAACAC -3'	5'- CAGAATCCAC AGCATGGCGAA CAGAAACACG -3'

Supplementary Table 4. Solutions used for acute slice in vitro electrophysiology

All compounds used are listed with their concentrations. QX314 stands for N-(2,6-dimethylphenylcarbamoylmethyl) triethylammonium chloride, an intracellular sodium channel blocker.

<i>aCSF 2mM Ca²⁺</i>		<i>Intracellular solution</i>	
Compound Name (Source)	Concentration (mM)	Compound name (Source)	Concentration (mM)
Glucose (Merck)	13	K-gluconate (Merck)	108
NaCl (Merck)	125	HEPES (Merck)	9
CaCl ₂ (Merck)	2	EGTA (Merck)	9
MgCl ₂ (Merck)	1	Na ₂ Phosphocreatine (Merck)	14
NaHCO ₃ (Merck)	26	ATP-Mg (Merck)	4
NaH ₂ PO ₄ * H ₂ O (Merck)	1.25	GTP-Na (Merck)	0.3
KCl (Merck)	2.5	MgCl ₂ (Merck)	4.5
Na-Pyruvate (Merck)	2	QX-314 (Merck)	1
Na L-ascorbate (Merck)	0.7		

<i>Cutting Solution</i>	
Compound Name (Source)	Concentration (mM)
Glucose (Merck)	20
Sucrose (Merck)	120
NaCl (Merck)	50
CaCl ₂ (Merck)	0.2
MgCl ₂ (Merck)	6
NaHCO ₃ (Merck)	26
NaH ₂ PO ₄ * H ₂ O (Merck)	1.25
KCl (Merck)	2.5
Na-Pyruvate (Merck)	2
Na L-ascorbate (Merck)	0.7
Na L-Lactate (Merck)	3