High frequency neural spiking and auditory signaling by ultrafast red-shifted optogenetics

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Optogenetics revolutionizes basic research in neuroscience and cell biology and bears potential for medical applications. We develop mutants leading to a unifying concept for the construction of various channelrhodopsins with fast closing kinetics. Due to different absorption maxima these channelrhodopsins allow fast neural photoactivation over the whole range of the visible spectrum. We focus our functional analysis on the fast-switching, red light-activated Chrimson variants, because red light has lower light scattering and marginal phototoxicity in tissues. We show paradigmatically for neurons of the cerebral cortex and the auditory nerve that the fast Chrimson mutants enable neural stimulation with firing frequencies of several hundred Hz. They drive spiking at high rates and temporal fidelity with low thresholds for stimulus intensity and duration. Optical cochlear implants restore auditory nerve activity in deaf mice. This demonstrates that the mutants facilitate neuroscience research and future medical applications such as hearing restoration.
Microbial-type rhodopsins, light gated cation channels (Channelrhodopsins, ChRs) and light-driven ion pumps are useful tools for multimodal optogenetic control of electrically excitable cells in culture, tissue and living animals1-3. Since the first description of the ChRs in 2002 and 2003, a set of different ChRs including red-shifted variants like VChR1, ReaChR and Chrimson have been described4-8. For different purposes ChRs were modified with respect to the kinetics, ion selectivity as well as light absorption9-11. ChR kinetics is a major issue, because the light sensitivity is regulated via the open lifetime of the channel12. Channels with a short open lifetime need correspondingly stronger light than channels with a long open lifetime for maximal photostimulation. This is due to the essential invariance of other channel parameters like single channel conductance and quantum efficiency. The mutual dependence between channel kinetics and light sensitivity accounts for the optimization of ChR expression and light delivery for successful experiments in the high frequency range. Although fast channels need stronger light for the activation, high speed is indispensable for many optogenetic applications in neurobiology because many types of neurons operate at high firing rates in the intact animal.

Prominent examples include spiral ganglion neurons (SGNs) of early auditory pathway and fast spiking interneurons in cortical areas, which fire action potentials at up to several hundred Hz13,14. However, light stimulation of ChR2-expressing SGNs indicated a strong limitation of the temporal response fidelity15. Therefore, fast ChRs are needed and their benefit for use in auditory research has already been indicated using Chronos, a “fast” blue light absorbing ChR, for stimulation of the cochlear nucleus16.

Electrical cochlear implants (eCI), to date, enable speech understanding in most of approximately 500,000 otherwise deaf users. However, the bottleneck of eCI is the poor frequency resolution of coding that results from wide current spread from each electrode contact and limits speech understanding in background noise17. Optical cochlear implants (oCI)–stimulating optogenetically modified SGNs, promise a fundamental advance of prosthetic sound coding by increasing frequency resolution, because light can be better confined than the electric field of electrodes18. For oCI eventually to be translated into the clinic, because light can be better confined than the electric field of electrodes, opsins need to be delivered into the SGNs by postnatal virus application to the ear and should endow SGNs with high light-sensitivity and temporal fidelity of spike generation, while light scattering and blue light induced phototoxicity should be minimized. Due to the aforementioned, adverse effects of optogenetic stimulation using blue light, the already available, fast blue light-activated ChR variants like ChETA (τoff = 4.4 ms) and Chronos (τoff = 3.6 ms) might have a limited applicability in animals and future clinical translation.

Here, we report that fast gating can be generally conferred to ChRs by helix 6 (helix F) mutation and demonstrate the utility of fast red-shifted ChRs for driving spiking of fast cerebral interneurons to the limit of their encoding range. Moreover, we established efficient virus-mediated delivery and expression of a fast Chrimson mutant in SGNs of mice, show that single-channel oCIs enable near-physiological spike rates and spike timing in SGNs and restore auditory activity in deaf mice. We demonstrate on several cell types in vitro and in vivo that the unfavorable low light sensitivity for activation is compensated by high expression levels of the fast Chrimson mutants.

Results
Fast helix F mutants and their calcium permeabilities. Closed to open state transition is associated with movement of helix F in several microbial-type rhodopsins19-21. Thereby helix F movement controls protonation reactions during vectorial proton transport and consequently the cycle time22,23. Closed to open state transitions of helix F have recently been verified for ChR 224,25. Motivated by these findings we performed a systematic study about the effects of helix F mutations on the closing kinetics of ChR (Fig. 1). We heterologously expressed ChRs helix F mutants in neuroblastoma-gliaoma cells (NG cells) and performed whole-cell patch-clamp experiments. The helix F mutant F219Y significantly accelerated the closing kinetics of ChRs 2 (Fig. 1c and Table 1). Mutations at the homologous positions of VChR1 (F214Y), ReaChR (F259Y) and Chrimson (Y261F) also accelerated the closing kinetics (Fig. 1b), albeit to a different extent (Fig. 1d-f and Table 1). The strongest effect on the lifetime of the channels was observed in ReaChR and VChR1, where the closing kinetics is accelerated by one order of magnitude (Table 1).

Interestingly, the relative calcium permeability of ChR2 F219Y PCa/PNa = 0.30 ± 0.02 (n = 4) was increased compared to the relative calcium permeability of ChR2 wt PCa/PNa = 0.13 ± 0.01 (n = 4). Permeability ratios were calculated according to the Goldman–Hodgkin–Katz equation26 with the measured values of the reversal potentials after replacing external sodium by calcium. The critical role of a tyrosine at the homologous position on the calcium permeability is verified in ReaChR and Chrimson (Supplementary Table 1). Of note F219 (ChR2 numbering) points to L132 (ChR2 numbering) on helix C in the chimera C1C2 crystal structure (Fig. 1a). ChR2 L132C has an increased calcium permeability (CatCh, calcium translocating ChRs)27. In contrast to the FY mutations on helix F, which accelerate the closing kinetics the L132C mutation (helix C) as well as the corresponding mutations at the homologous positions of VChR1, ReaChR and Chrimson significantly slowed the closing kinetics (Table 1). Structural information, the effect on the kinetics and the effect on the calcium permeability indicate a probable interaction of helix C and helix F at those critical residues.

Chrimson mutants with accelerated closing kinetics. As shown above the Y261F mutation speeds up channel closing in Chrimson (Fig. 1f, Table 1). We identified two additional helix F mutations, which accelerated Chrimson’s closing kinetics, namely S267M and Y268F (Fig. 1b, g and Table 1). The combination of the helix F mutations had a cumulative effect, further accelerating channel closing by up to one order of magnitude (Fig. 1g and Table 1). Chrimson mutants carrying the Y268F mutation showed reduced expression in NG cells and a hypochromic shift of their action spectra by 11 nm (Supplementary Fig. 1 and Supplementary Table 2). The hypochromic shift might result from an interaction of F268 with the polyene chain of the retinal, as this was shown for F226, ChR2-numbering, located at the homologous position in the C1C2 structure (Fig. 1a).

Of special interest for optogenetic applications are the fast mutant Chrimson Y261F/S267M (f-Chrimson) and the very fast mutant Chrimson K176R/Y261F/S267M (vf-Chrimson), which carries the additional K176R mutation (Fig. 1h). As described earlier3, the closing kinetics of Chrimson K176R (ChrimsonR) is accelerated by a factor of ~2 compared to wildtype (Table 1). The closing kinetics of f-Chrimson were strongly accelerated from τoff = 24.6 ± 0.9 ms (wt-Chrimson, n = 5) to τoff = 5.7 ± 0.5 ms (f-Chrimson, n = 5). At the same time f-Chrimson was highly expressed in NG cells (Supplementary Table 2). Vf-Chrimson had ultrafast closing kinetics of τoff = 2.7 ± 0.3 ms (n = 7), which is at least as fast as the closing kinetics of Chronos.
6, the fastest ChR known to date. Of note, the action spectra of f-Chrimson and vf-Chrimson were not blue-shifted (Supplementary Fig. 1), thereby preserving the benefits of longer wavelength activation. Compared to Chrimson wt, the functional properties of the mutants were almost unaltered with respect to the linear voltage dependence of the photocurrents (Supplementary Fig. 2), cation permeabilities (Supplementary Table 3), the moderate slowing of the closing kinetics at positive voltages (Supplementary Fig. 3) and peak current inactivation (Supplementary Fig. 4). Of note, the measured cation permeabilities contradict a recent publication26, but are in accordance with a previous study6. At a temperature of 34 °C we measured $\tau_{\text{off}}$ values of 3.2 ± 0.2 ms ($n = 3$) for f-Chrimson and 1.6 ± 0.1 ms ($n = 3$) for vf-Chrimson (Supplementary Fig. 5). Hence the ultrafast kinetics of the Chrimson mutants in principle enables neural photostimulation in an exceptionally high frequency range of up to ~600 Hz.

**Ultrafast red-shifted optogenetics.** We heterologously expressed f-Chrimson and vf-Chrimson in primary cultures of rat hippocampal neurons by means of adeno-associated virus-mediated gene transfer (AAV2/1). Patch-clamp experiments proved a robust neuronal expression and confirmed the substantially faster kinetics of the mutants (Supplementary Table 4). The application of light pulses ($\lambda_1 = 594$ nm, $\lambda_2 = 640$ nm) triggered spiking with high reliability (Fig. 2 and Supplementary Fig. 7). The investigation of the dependence of spike probability on light pulse intensity showed that neural photostimulation via f-Chrimson (0.37–1.27 mW/mm²) and vf-Chrimson (0.09–3.18 mW/mm²)
f-Chrimson is a promising candidate for hearing restoration. Optogenetics bears great potential for improving the restoration of vision and hearing.\textsuperscript{28,29} Future oCIs shall use tens to hundreds of microscale light sources to focally stimulate tonotopically-ordered SGNs in Rosenthal’s canal. For deaf people, the lower spread of excitation from the light source in oCIs\textsuperscript{15} promises improved frequency and intensity resolution when compared to the eCI\textsuperscript{28}. However, much remained to be done prior to a potential clinical translation of the oCI. For example, so far cochlear optogenetics was established using blue ChRs expressed in transgenic rodents or in mice following prenatal viral-gene transfer\textsuperscript{15}.

Here, we tested the potential of f-Chrimson for optogenetic stimulation of SGNs. We established postnatal viral gene transfer by injecting AAV2/6-hSyn-f-Chrimson-EYFP into the scala tympani via the round window in 3–6–day-old mice (Fig. 4b). We readily observed photocurrents in patch-clamp recordings from isolated SGNs\textsuperscript{30} in the second postnatal week (Fig. 4c), proving the basic functionality of f-Chrimson in the target cells. We then in depth analyzed expression and function 4–14 weeks after injection. Using confocal imaging of EYFP and parvalbumin immuno-fluorescence in cochlear cryosections we found a high transduction rate (near 80 %) in the injected ear, which was not significantly different between the cochlear turns (Kruskal–Wallis ANOVA followed by Dunn’s test, \(P > 0.05\), \(n = 5\); Fig. 4d, f). SGN showed clear plasma membrane expression of f-Chrimson (insets in Fig. 4f) and survived the optogenetic manipulation as evident from the unaltered SGN density when compared to the non-injected ear (Mann–Whitney U test, \(P > 0.05\), \(n = 5\); Fig. 4e, f). The non-injected ear showed f-Chrimson expression in less than 5% of the SGNs (Fig. 4d, f), indicating minimal spread of AAV from the injected ear likely via the cochlear aqueduct.

We then established single-channel oCI stimulation by performing a posterior tympanotomy and inserting an optical fiber (50 µm diameter) through the round window to project the light of a 594 nm laser onto the SGNs of the basal cochlear turn of young mice (2–3 months, Fig. 5a). We could readily elicit optical auditory brainstem responses (oABR, Fig. 5b, c) that differed between animals in waveform and amplitude. For comparison we
recorded acoustic auditory brainstem responses (aABRs, Fig. 5b, c lower panels) that were similar in amplitude and waveform to oABR and also varied between animals (Fig. 5b). We note that the similarity to aABRs and the shorter latency (0.93 ± 0.13 ms vs. approximately 3 ms) and smaller maximal amplitude (10.7 ± 3.80 µV vs. approximately 1000 µV) indicates more specific activation of the auditory pathway in the case of postnatal AAV-injection used in present study. We then characterized the oABRs in response to different light intensities, light pulse durations and light pulse rate (n = 5 mice). oABR amplitude grew and oABR latency got shorter with increasing light intensity (Fig. 5c, d, g). Stimuli as weak as 0.5 mW (Fig. 5c,d, duration: 1 ms, rate: 20 Hz) and as short as 80 µs (Fig. 5e, h, rate: 20 Hz, intensity: 11 mW) were sufficient to drive oABRs. Amplitudes typically varied for changes in light intensity of more than one order (Fig. 5c, d, output dynamic range >20 dB for oABR). oABR amplitudes declined when raising stimulus rates (Fig. 5f, i). However, f-Chrimson-mediated oABRs remained sizable up to stimulus rates of 200 Hz, suggesting high temporal fidelity of light-driven SGN firing.

Next, we used aged C57BL6/J mice (9 months-old, n = 5 mice) to explore the potential of oCI to restore activity in the auditory pathway of a mouse model of age-related hearing loss, which is a major form of hearing impairment in humans. Auditory thresholds, estimated by aABR elicited by acoustic clicks were elevated to above 50 dB (SPL) (58 ± 3.3 dB SPL, Fig. 5j–l, typically 20 dB in young mice) and aABR amplitudes were reduced to 1/3 of those in young mice across all SPLs tested (Fig. 5l). oABR amplitudes measured in these aged mice were comparable to values obtained for young animals (Fig. 5l), but latencies tended to be shorter and less variable (Fig. 5g and Supplementary Fig. 10h). Interestingly, we found that light pulses as short as 40 µs were able to elicit oABRs (11 mW at 20 Hz, Supplementary

Fig. 2 Light-induced spiking in rat hippocampal neurons.  
(a–d) Spiking traces at different light-pulse frequencies. Rat hippocampal neurons heterologously expressing Chrimson-EYFP (a), Chrimson-EYFP K176R/Y261F/S267M (vf-Chrimson-EYFP) (b) and Chrimson-EYFP Y261F/S267M (f-Chrimson-EYFP) (c, d) were investigated by whole cell patch-clamp experiments under current-clamp conditions (λ = 594 nm, pulse width = 3 ms, saturating intensity of 11–30 mW/mm²). 
(e) Traces from two different cells at a stimulation frequency of 60 Hz. 
(f) Traces from one cell at stimulation frequencies of 80 Hz and 100 Hz. 
(g) The dependence of spike probability on light pulse intensity for Chrimson-EYFP (e) (15 different cells), Chrimson-EYFP Y261F/S267M (f-Chrimson-EYFP) (f) (15 different cells) and Chrimson-EYFP K176R/Y261F/S267M (vf-Chrimson-EYFP) (g) (15 different cells). The action potentials were triggered by 40 pulses (λ = 594 nm, pulse width = 3 ms, ν = 10 Hz) of indicated light intensities. In order to determine the spike probability, the number of light-triggered spikes was divided by the total number of light pulses. Scale bars: y-axis: 10 mV, time-axis: (a, b, 10 Hz) 500 ms (a, b, 20 Hz) 300 ms (c, 60 Hz) 100 ms (d, 80 Hz) 70 ms (d, 100 Hz) 50 ms
Moreover, we were able to record oABRs at stimulation frequencies as high as 250 Hz (11 mW, 1 ms pulse duration), likely due to the lower average latencies found in these aged mice as compared to their younger counterparts (Supplementary Fig. 10g, j). Together the data indicate that optical activation of the auditory pathway proceeded with at least as high efficiency in aged C57BL/6J mice despite their profound age-related hearing impairment. f-Chrimson expression levels throughout the injected cochlea were homogeneous (one-way ANOVA followed by Tukey’s test, \( P > 0.05, n = 5 \)). Importantly, long-term f-Chrimson expression (9 months) did not seem to decay significantly (Kruskal–Wallis ANOVA followed by Dunn’s test, \( P > 0.05, n = 5 \)) (Supplementary Fig. 10a–c) nor cause any significant loss of SGNs in the AAV-injected ear of these mice, when compared to the non-injected ear (t-test for comparison of

**Fig. 3** f-Chrimson drives fast interneurons to the frequency limit. a Example recording of a neocortical parvalbumin-positive interneuron in an acute brain slice. Current injection (500 ms, 550 pA) elicits high frequency firing (322 Hz), consistent with the fast spiking phenotype of these interneurons. b When tested with constant current injection, the input-output curve of PV-interneurons plateaus at a maximum firing rate of 270 ± 33 Hz (n = 8). c Example traces of the f-Chrimson-expressing PV-interneuron from a activated by light pulses (565 nm, 0.5 ms) at frequencies ranging from 50–500 Hz. Note that this interneuron reliably followed frequencies of up to 400 Hz. d Spiking probabilities of PV-interneurons at different optical stimulation frequencies. On average (black), PV-interneurons followed stimulation up to 300 Hz reliably (94 ± 5% spiking probability), and could still encode input frequencies of up to 400 Hz with a reliability of 68 ± 16% (n = 7; three whole-cell, four cell-attached recordings). e Action potential latency (assessed at peak) and action potential jitter (s.d. of latencies) after light pulse onset for all stimulation frequencies with reliable spiking (>85%). Error bars are s.e.m. Scale bars: a 50 ms, 10 mV c 50 ms, 10 mV

Fig. 10f, i) as compared to 80 µs in young mice (Fig. 5e).
cell density across cochlear turns in the injected and non-injected ear, \( P > 0.05, n = 5 \) (Supplementary Fig. 10d).

In order to scrutinize the temporal fidelity of stimulation, we turned to juxtacellular recordings from single neurons\(^{13,33}\). We established single-channel oCI stimulation via an optical fiber and targeted electrodes through a craniootomy to where the auditory nerve enters the cochlear nucleus (Fig. 6a) in order to measure the neural photoactivation. Those neurons could not be identified based on a response to acoustic stimulation, most likely due to impaired acoustic hearing following ear surgery and oCI. Therefore, we termed light-stimulated neurons putative SGNs.

We found that the putative SGNs fired upon optogenetic stimulation with high temporal precision for stimulus rates of up to hundreds of Hz (Fig. 6b–e): some neurons followed stimulation to some extent even up to 1 kHz (Fig. 6b, d). The spike latency amounted to approximately 2 ms for stimulus rates up to 400 Hz (Supplementary Fig. 11a, b), which is in agreement with the data obtained on the interneurons (Fig. 3e). Temporal precision of firing, evaluated based on vector strength (ref. \(^{34}\), see methods, Fig. 6c, d) and temporal jitter (i.e., standard deviation of spike latency across trials, Fig. 6e, Supplementary Fig. 11c) varied between the recorded neurons and, generally, was good. The vector strength declined with increasing stimulation rate up to 1 kHz. For a comparison, we re-plot the median vector strength of firing driven by transposed tones in mouse SGNs (ref. \(^{35}\), Fig. 6d) used because phase-locking to pure tones is hard to achieve in the high frequency mouse cochlea\(^{36}\). Temporal jitter, evaluated for spikes occurring in the time window equal to the period stimulus, was typically below a millisecond and tended to increase when raising stimulus rates up to 300 Hz (Fig. 6e). At higher stimulus rates, the temporal jitter was higher than the values obtained for simulated Poisson spike trains (see methods, gray area, Fig. 6e), reflecting a reduced spike synchronization with the light pulses. Interestingly, the spike jitter increased significantly from 25 ms compared to the start of the stimulation (Supplementary Fig. 11c). Spike probability (Fig. 6c, d) declined
**Fig. 5** Single-channel oCIs drive oABRs in hearing and deaf mice. 

**a** Experimental set-up for oABR-recordings in mice: a 50 µm optical fiber coupled to a 594 nm Obis laser was implanted into scala tympani via a posterior tympanotomy and the round window. Recordings of far-field optically evoked potentials were performed by intradermal needle electrodes. For aABR recordings a free-field speaker was employed (lower panel). 

**b** Comparing oABRs (upper panel) and aABRs (lower panel) at strong stimulation levels for four mice (average of 1000 trials). oABRs were recorded in response to 1 ms long, 11 mW, 594 nm laser pulse at 10 Hz, aABRs of the same mice in response to 80 dB (SPL peak equivalent) clicks. Bars indicate the stimulus timing. 

**c** oABRs (upper panel, 594 nm, 1 ms at 10 s⁻¹) and aABRs (lower panel, clicks at 10 s⁻¹, values in SPL [peak equivalent]) recorded from an exemplary AAV-injected mouse at increasing stimulus intensities. 

**d**–**f** Normalized P1-N1-amplitude as a function of laser intensity (d 1 ms at 20 Hz), pulse duration (e 11 mW at 20 Hz), and stimulus rate (f 11 mW, 1 ms). Group average (lines) and s.d. (error bars) are shown in orange (same for **g**–**i**). 

**g**–**i** P1-latency as a function of laser intensity (g as in **d**), duration (h as in **e**), and rate (i as in **f**). 

**j** Exemplary aABR recordings done as in **a**–**c** using a 9 months-old mouse (following postnatal AAV-Chrimson-EYFP injection: elevated acoustic thresholds (around 60 dB [SPL], compare to **c**). 

**k** oABR recordings done as in **a**–**c** in the same mouse as in **j**, using 1 ms long laser pulses: thresholds similar to injected mice at 2–3 months of age (around 1 mW, compare to **c**). 

**l** P1-N1-amplitude of oABR (orange) and P1-N1-amplitude of aABR (gray) as function of stimulus intensity in young (2–3 months-old) and old (9 months-old) mice (n = 5 for each group, means (lines) ± s.e.m. (error bars) are shown. Symbols in **d**–**l** mark results from individual animals. Scale bars (**b**, **c**, **j**, **k**): 1 ms, 5 µV
as the rate of stimulation increased, indicating that optogenetic coding by individual SGNs becomes less reliable as stimulus rate rises. This, however, is likely compensated at the population level, as several SGNs jointly encode information from each place of the tonotopic map.

**Discussion**

As demonstrated, the investigation of the molecular properties of microbial-type rhodopsins is essential for the development of variants with superior properties for particular optogenetic applications. Our study reveals the critical role of helix F for the closing kinetics of various ChRs. Using site-directed mutagenesis we generated fast variants of four different ChRs, which, together, cover photoactivation over the visible spectrum. We deem the fast (f) and very fast (vf) Chrimson of particular interest to the neurosciences because of their red-shifted action spectrum and high membrane expression. Our analysis of fast spiking interneurons of the cerebral cortex demonstrated that they enable the remote optical control of even the fastest neurons at their intrinsic physiological limits. Finally, we show that f-Chrimson is...
a promising candidate for future clinical optogenetic restoration of sensory function.

Channel opening/closing of ChR2 is based on a concerted movement of helices B, F, and G. Interestingly, we discovered a major impact of the interaction between the moving helix F and the virtually immobile helix C on ChR kinetics. High-resolution structures of the investigated ChRs are not available. However, the information of the high-resolution structure of the CIC2 ChR chimera in combination with the light-induced helix movement studies by electron spin resonance and the low-resolution structure by 2D cryoelectron microscopy allowed us to identify the crucial position F219 in helix F for the construction of a faster ChR2 mutant. This position is also conserved in ReaChR and VCR1. Analogous mutations lead to accelerated kinetics of channel closing. Of note, the surprisingly slow reacting Chrimson has already a tyrosine on this position (Fig. 1a, b). We suspected that the mutation of this tyrosine influences channel closing kinetics. Indeed, the mutation to a phenylalanine in the analogous position (Y261F) results to faster channel closing in Chrimson. Further inspection of the alignment of helix F shows at positions M225 and F226 in ChR2 the same analogy for VCR1 and ReaChR but not for Chrimson. For Chrimson, mutations on these positions (S267M, Y268F) in addition to the Y261F mutation result in the ultrafast switching behavior (Fig. 1g,h). The surprising and peculiar phenotype of the back mutations in Chrimson is hard to explain without a high resolution structure of the protein.

This study achieved two important breakthroughs towards developing cochlear optogenetics for auditory research and future improved hearing restoration. First, we managed to achieve efficient, non-traumatic and neuron-specific expression of f-Chrimson in SGNs using postnatal AAV-injection into scala tympani through the round window. We found near 80% of the SGNs of the injected ear to express f-Chrimson at high levels and mostly in the plasma membrane of somas and neurites, which persists for at least 9 months after injection. These transduction rates were much higher than those achieved with transurethral injection of AAV2/6-hSyn-CatCh-YFP and unlike there, independent from tonotopic position. We consider the minute transduction of the non-injected ear to reflect viral spread through the cerebrospinal fluid space, which calls for further optimization of the injection protocol. Importantly, we did not find evidence for neuron loss even at 9 months after injection and we expect little, if any, risk of phototoxicity due to the red-shifted action spectrum of f-Chrimson.

Secondly, using f-Chrimson, we overcame the likely biggest roadblock of current ChR2-based cochlear optogenetics: low temporal bandwidth of optical coding (<80 Hz). We found robust and fast photocurrents in cultured isolated f-Chrimson-positive SGNs. In vivo, fiber-based stimulation resembling single-channel oCI elicited activation of the auditory pathway in hearing and deaf mice. Using far-field neural population responses (oABR) as a readout we found low thresholds for radiant flux and energy (<0.5 mW, <0.5 μl) as well as duration (<100 μs) and a wide dynamic range of coding (more than 20 dB, no saturation for most animals at maximal stimulation). This brings the oCI closer to the currently used eCI stimulation parameters (0.2 μl and 80 μs per pulse) and exceeds the eCI output dynamic range (<10 dB). Both, recordings of oABR and of firing in single putative SGNs indicated that f-Chrimson mediated oCI-enabled responses to follow at least 200 Hz, which corresponds to physiological steady-state firing rates of SGNs. In fact, we found some neurons to follow stimulation to several hundreds of Hz nearly mimicking sound-evoked SGN activity. The closing kinetics of f-Chrimson and the resulting relative refractoriness probably also limits the temporal precision of f-Chrimson-mediated SGN firing. At 500 and 1000 Hz, vector strength, a measure commonly used to analyze the extent of phase-locking in SGNs, was lower for f-Chrimson-mediated optogenetic stimulation than for mouse SGN firing with transposed tones. We note that the SGNs recorded in the present study typically did not fire spontaneously probably due to the ear surgery. Besides the lack of spontaneous firing, the short (1 ms) and pulsatile optogenetic stimulation typically evoking a single spike likely explain why vector strength tended to be higher for low stimulus rates when compared to transposed tones, for which several spikes were generated per stimulus cycle. Moreover, vector strength and temporal jitter of f-Chrimson-mediated SGN firing in mice indicate a lower temporal precision than that of acoustic hearing and electric stimulation in species with prominent phase-locking of SGN firing. Nonetheless, we reason that, even if the limited probability and temporal precision of single SGN firing for optogenetic stimulation at 100–500 Hz translated to species other than the mouse, this will not impede the coding at the level of the auditory nerve population. Hence, we conclude that f-Chrimson is a good candidate opsin for the oCI. In fact, higher temporal resolution in response to optogenetic stimulation might render unnecessary the very high stimulation rates employed in eCI to avoid overly synchronized activity in the auditory nerve. The favorable properties of the novel Chrimson mutants also facilitate multiple applications in basic neurosciences and in sensory restoration, such as the recovery of vision.

Methods

Molecular biology. The humanized DNA sequence, coding for the red light activated ChR Chrimson from Chlamydomonas reinhardtii (accession number: KF992060), either C-terminally fused to EYFP or without a fluorescent tag, was cloned into the mammalian expression vector pcDNA3.1–(−) (Invitrogen, Carlsbad, USA). The mutations L174C, K176R (ChrimsonR), Y261F, S267M and Y268F as well as combinations of aforementioned mutations (Table 1) were created by site-directed mutagenesis. Chrimson-EYFP wt and Chrimson-EYFP Y261F/S267M (f-Chrimson-EYFP) were subcloned into the Xenopus laevis oocyte expression vector pTLN40.

We also cloned the humanized DNA sequences coding for ChR2 (C-terminally truncated variant Chop2-315 of ChR2 from Chlamydomonas reinhardtii, accession number: AF461397), for Volvox ChR1 (VChR1, accession number: EU622855) and for the chimera ReaChR (ChR1/VChR1/VChR2, Red-activatable ChR, accession number: KF448069) into the mammalian expression vector pcDNA3.1–(−) (Invitrogen, Carlsbad, USA). Thereby Chr2 and VChR1 were C-terminally fused to EYFP and ReaChR was C-terminally fused to Citrine. The mutants ChR2-EYFP F219Y, VChR1-EYFP F214Y, VChR1-EYFP L127C, ReaChR-Citrine F259Y and ReaChR-Citrine L172C were created by site-directed mutagenesis. The related primer sequences were shown in Supplementary Tables 5 and 6.

NG108-15 cell culture and transfection. NG108-15 cells (ATCC, HB-12377TM, Manassas, USA) were cultured at 37 °C and 5% CO2 in DMEM (Sigma, St. Louis, USA) supplemented with 10% fetal calf serum (Sigma, St. Louis, USA), and 5% penicillin/streptomycin (Sigma, St. Louis, USA). One day prior to transient transfections the NG108-15 cells were seeded on 24-well plates. Two to three days prior to their electrophysiological characterization by patch-clamp experiments the NG108-15 cells were transiently transfected with pcDNA3.1–(−) derivatives carrying aforementioned Chrs and ChR mutants using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) or Lipofectamine LTX (Invitrogen, Carlsbad, USA). Cells were tested for mycoplasma contamination using specific primers. No method of cell line authentication was used.

Expression of Chrimson variants in Xenopus laevis oocytes. Xenopus laevis oocytes were injected with 50 ng of in vitro-transcribed cRNA (Thermo Fisher Scientific, Waltham, USA), coding for Chrimson-EYFP wt and Chrimson-EYFP Y261F/S267M (f-Chrimson-EYFP). After cRNA injection the Xenopus laevis oocytes were incubated at 16 °C in an 1 μl all-trans retinal containing Barth’s solution (88 mM NaCl, 1 mM KCl, 0.33 mM Na(HCO3)2, 0.41 mM CaCl2, 0.82 mM MgSO4, 2.4 mM NaHCO3, 10 mM HEPES, pH 7.4 supplemented with 50 mg/ml gentamicin, 67 mg/ml penicillin and 100 mg/ml streptomycin) for 4–5 days.

Electrophysiological recordings on Xenopus laevis oocytes. The Xenopus laevis oocytes heterologously expressing the Chrimson variants were investigated by the two electrode voltage-clamp technique. Photocurrents were measured in response to 500 ms light pulses with a wavelength of λ = 590 nm using the LED.
Resistance ranged from 1.1 to 4.6 GΩ. The series resistance was <10 MΩ.

The intracellular solution contained 110 mM KCl, 2 mM MgCl₂, 5 mM HEPES, 10 mM EGTA, 10 mM HEPES, pH 7.4 and the bath solution contained 140 mM NaCl, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM HEPES, pH 7.4.

In order to assess the permeability of potassium ions relative to the permeability of sodium ions (PNa/PK), we measured photocurrents at voltages ranging from −120 to +40 mV in 20-mV steps. The PNa/PK ratio was determined from the difference of the reversal potentials of the photocurrents when replacing 90 mM NaCl, 2 mM MgCl₂, 5 mM MOPS/TRIS pH 9 for 90 mM KCl, 2 mM MgCl₂, 5 mM MOPS/TRIS pH 9.

The series resistance was determined using a fast capacitive transient technique with a slow decay curve. The decay time constant was determined by the capacitance of the cell. In order to determine the lowest light intensity required to induce action potentials with a probability of 100% (I50), 40 pulses (λ = 594 nm, pulse width = 3 ms, v = 10 Hz) of varying light intensities were applied. The spike probability was calculated by dividing the number of light-triggered spikes by the total number of light pulses.

Electrophysiological recordings on hippocampal neurons. For whole-cell recordings in cultured hippocampal neurons, patch pipettes with resistances of 3–8 MΩ were filled with 129 mM potassium gluconate, 10 mM HEPES, 10 mM KCl, 4 mM MgCl₂, 0.3 mM Na₂GTP, and 30 mM glucose and 25 mM HEPES, titrated to pH 7.4. The series resistance was <10 MΩ and the input resistance ranged from 0.7 to 3.5 GΩ. The mean capacitance of the measured cells was 35.4 ± 12.4 pF (n = 31). In order to avoid an experimental bias in cell selection, the neurons for the electrophysiological recordings were selected independent of the brightness of their EYFP fluorescence. Recordings were conducted in the presence of the excitatory synaptic transmission blockers, 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide (NBOX, 10 µM, Sigma-Aldrich, St. Louis, USA) and D(-)-2-Amino-5-phosphopentanoic acid (AP5, 20 µM, Sigma-Aldrich, St. Louis, USA). For determination of the OFF kinetics, measurements were conducted in the presence of 1 µM TTX (Sigma-Aldrich, St. Louis, USA) in addition. Electrophysiological signals were amplified using an Axopatch 200B amplifier (Axon Instruments, Union City, USA), filtered at 10 kHz, digitized with an Axon Digidata 1322 A (50 kHz) and acquired and analyzed using pClamp6 software (Axon Instruments, Union City, USA).

The light pulses had a pulse width of 3 ms, a wavelength of λ = 594 nm and a membrane capacitance of 11–30 mF/m². The t50 value was determined by a fit of the decaying photocurrent to a monoexponential function. The current density (J(t))/V(t) was determined by subtracting the saturating intensity of 23 mW/mm² using diode-pumped solid-state lasers. If not stated differently the OFF kinetics was determined at room temperature (297 K). The temperature dependence of the OFF kinetics of Chrimson-EYFP wt and mutants were investigated at a membrane potential of −70 mV, 60 mV.

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Enzymatic digestion of the hippocampal tissue was performed in accordance with institutional guidelines and were approved by the Regierungspräsidium Darmstadt.

Intracerebroventricular injections. Prior to pup injections, the dam was habituated to the experimenter and the experimental room. Newborn mice (P2) were anesthetized using isoflurane (2–3%), and placed on a light source to reveal skull structures. Injections of 2 µl of AAV2/1-Ishyv-vf-Chrimson-EYFP were performed into the right ventricle using a glass pipette (coordinates from bregma: rostral 0.75 mm, lateral 0.25 mm and ventral 2 mm). After injection pups were recovered for 5 min in a pre-warmed container with homecage-bedding before being placed back in the home cage.

Patch-clamp recordings on parvalbumin-positive interneurons. Coronal brain slices were prepared from 2–6-week old PV-tDTomato mice that had been injected with 2 µl of AAV2/1-Ishyv-vf-Chrimson-EYFP at postnatal day 2. Animals were anesthetized with isoflurane (3% in oxygen), decapitated and the brain was dissected in cold artificial cerebrospinal fluid (ACSF) containing in mM: 125 NaCl, 3 KCl, 2 CaCl₂, 1 MgCl₂, 26 NaHCO₃, 10 glucose, and sliced (325-µm thick) on a vibrating microtome (VT1205S; Wetzlar, Germany) at 4 °C. Slices were recovered for 60 min at room temperature in a submersion chamber containing ACSF equilibrated with 95% O₂/5% CO₂. Slices were next transferred to the submersion chamber of an upright microscope (Scientifica), and continuously superfused with ACSF additionally containing 1 mM DNQX, 40-µM AP5 and 1-µM bicuculline at 33 °C. Parvalbumin-positive interneurons were identified in layer 2/3 of neocortex with a combination of infrared and fluorescence video microscopy under a 40x objective (Olympus). Patch-clamp electrodes (8-12 MΩ) were pulled from borosilicate glass and filled with an intracellular solution consisting of (in mM): 140 potassium–glucuronate, 10 HEPES, 4 phosphocreatineNa₂, 4 Mg-ATP, 0.4 Na-GTP, 10 KCl (pH adjusted to 7.25 with KOH, –280–300 Osm). Data were acquired with a Multiclamp700B amplifier and pClamp 10.2 software (Axon Instruments).

Optogenetically evoked action potentials were recorded in parvalbumin-positive interneurons that had been infected with the lentivirus and recorded in the loose-seal cell-attached (n = 4) or whole-cell current-clamp (n = 3). In addition, another five parvalbumin-positive interneurons were recorded for the input–output curve presented in Fig. 3b. Data were filtered at 20 kHz and digitized at 20 kHz.
sampled at 50 kHz. Spiking patterns were assessed with depolarizing current steps in eight whole-cell recordings, and displayed the fast-spiking phenotype expected for PV-interneurons (n = 8). Intracellular filling (200–250 nl), stimulation was performed through the objective by a LED (coolLED) coupled to the microscope. Pulse width was 0.25–1 ms, irradiance ranged from 1–10 mW/mm², and was adjusted individually for each neuron to cause reliable firing (Fig. 3c: 50 Hz, pulsewidth = 0.5 ms, light-density = 2.6 mW/mm²; 100 Hz, pulsewidth = 0.5 ms, light-density = 2.6 mW/mm²; 150 Hz, pulsewidth = 0.5 ms, light-density = 4.9 mW/mm²; 200 Hz, pulsewidth = 0.5 ms, light-density = 4.9 mW/mm²; 250 Hz, pulsewidth = 0.5 ms, light-density = 4.9 mW/mm²; 300 Hz, pulsewidth = 0.5 ms, light-density = 4.9 mW/mm²; 400 Hz, pulsewidth = 0.5 ms, light-density = 8.9 mW/mm²; 500 Hz, pulsewidth = 0.5 ms, light-density = 8.9 mW/mm²). Data were analyzed using clampfit and excel software. For calculation of latency and jitter, the time of action potential peak was used. We note that the apparent action potential threshold defined as the voltage at which the first temporal derivative crosses a threshold of 40 mV/s is more hyperpolarized for opto- genetically evoked action potentials (~57.0 ± 1.85 mV) compared to action potentials during DC current injections (~43.19 ± 2.97 mV, p < 0.001, unpaired, two-tailed t-test). Statistics were done using non-parametric Friedman test followed by a post-hoc Dunn’s test (Prism, GraphPad Software Inc., La Jolla, USA).

Cloning for AAV2/6 production. pcDNA3.1(−)–f-Chrisonm_EYFP was used as a starting material for cloning pAAV_hSyn_f-Chrisonm_EYFP. The sequence of f-Chrisonm_EYFP was amplified by means of a classical PCR. The resulting PCR fragment was then digested with BamHI/HindIII (Thermo Scientific, MA, USA) and gel extracted (GeneJET Gel Extraction Kit, Thermo Scientific, MA, USA) and further used for ligation. At the same time the plasmid pAAV_hSyn_Chronom_GFP (Addgene, plasmid nr. 59170) was also digested using restriction enzymes BamHI/ HindIII and used as a backbone plasmid. All obtained ligation products were further purified by precipitation of colony PCR and finally sequenced by an external company. The final product was then sent to the University of North Carolina Vector Core (Chapel Hill, NC, USA), and used to produce AAV2/6.

Postnatal AAV injection into the cochlea. All experiments were done in compliance with the German national animal care guidelines and were approved by the board for animal welfare of the University Medical Center Göttingen and the animal welfare office of the state of Lower Saxony. The calculation of animal number was performed prior to starting experiments. We planned to use the Wilcoxon Rank Sum Test and an error probability alpha smaller than 0.05, a power (1-beta) of 0.95 and effect size depending on the precise experimental protocol.

Postnatal AAV-injection into scala tympani of the left ear via the round window was performed at p3–p6 on C57BL/6 wild-type mice, using AAV2/6 and the human synapsin promoter to drive transgenic expression of f-Chrisonm-YFP in SGNs. In brief, under general isoflurane anesthesia and local analgesia achieved by means of xylazine, the left ear was approached via a dorsal incision and the round window membrane was identified and gently punctured using a borsilicate capillary pipette to allow access to the utricle and saccule. After virus application, the tissue above the injection site was repositioned and the wound was sutured and buprenorphine (0.1 mg/kg) was applied as pain reliever. Recovery of the animals was then daily tracked. Mice were randomly selected for injection in all experiments. No blinding was possible since injection was critically dependent on the right ear, which is the only existing auditory component. Hence, surgery prior to stimulation needed to be done in the injected ear. Animals were then kept in a 12 h light/dark cycle, with access to food and water ad libitum.

Immunostaining and imaging of cochlear cyrossections. Cochleae were fixed with 4% paraformaldehyde in phosphate buffered saline for 1 h. Cochleae were then cryosectioned following 0.12 M EDTA decalcification. After incubation of sections for 1 h in goat serum dilution buffer (16% normal goat serum, 450 mM NaCl, 0.6% Triton X-100, 20 mM phosphate buffer, pH 7.4), primary antibodies were applied for 1 h at room temperature. The following antibodies were used: chicken anti-GFP (catalog number: ab13970, dilution 1:300) (Abcam, Cambridge, United Kingdom), guinea pig anti-parvalbumin (catalog number: 195004, dilution 1:3000) (Synaptic Systems, Göttingen, Germany) and finally as following German manufacturer AlexaFluor-labeled antibodies were applied for 1 h at room temperature: goat anti-chicken 488 IgG (H + L), catalog number: A-11039, dilution 1:200 (Thermo Scientific, MA, USA); goat-anti guinea pig 586 IgG (H + L), catalog number A1107, dilution 1:200 (Thermo Scientific, MA, USA). Confocal images were collected using a SP5 microscope (Leica, Hamburg, Germany) and processed in ImageJ (NIH, Bethesda, MA, USA). Expression was considered positive when EYFP fluorescence in a given cell (marked by parvalbumin) was found to be higher than 3 SD above the background fluorescence of the tissue.

Animal surgery for recordings on the auditory pathway. Mice were anesthetized with i.p. administration of a mixture of xylazine (5 mg/kg) and urethane (1.32 mg/kg) and analgesia was achieved with buprenorphine. The core temperature was maintained constant at 37°C using a custom-designed heat plate on a vibration isolation table in a sound-proof chamber (IAC GmbH, Niederkirchen, Germany). For auditory nerve recordings, a tracheostomy was performed before the animals were positioned in a custom-designed stereotactic head holder. Pinnae were shaved, scalpel and reflected, perichondral bone removed, and a partial cerebellar aspiration performed to expose the surface of the cochlear nucleus.

Optical stimulation in vivo. The left bulla was reached using a retroauricular approach and opened to expose the cochlea. A 50 μm optical fiber coupled to a 594-nm laser (OBIS LS OPSL, 100 mW, Coherent Inc., Santa Clara, CA, USA) was inserted into the cochlea via the round window. Radiant flux was calibrated with a laser power meter (LaserCheck; Coherent Inc., Santa Clara, CA, USA).

SGN culture and patch-clamp recordings. On postnatal day 12–14, SGNs of mice were isolated, cultured and patch-clamped18. In brief, we patch-clamped EYFP-positive SGNs using an EPC-10 amplifier controlled by Patchmaster software (HEKA electronics, Lambrecht, Germany) and employing potassium-glucuronate based intracellular solution (in mM: 130 K-glucuronate, 5 KCl, 1 EGTA, 2 MgATP, 2 NaATP, 0.3 MgGtp, 10 KOH-HEPES, 10 Na2Phosphocreatinine) and an extracellular solution containing (in mM: 145 NaCl, 4 KCl, 1 MgCl2, 1.3 CaCl2, 10 NaOH-HEPES). A 594-nm laser (OBIS LS OPSL, 100 mW, Coherent Inc., Santa Clara, CA, USA) was coupled into a Nikon Eclipse inverted microscope and radiant flux was calibrated using a powermeter.

Auditory brainstem responses. For stimulus generation and presentation, data acquisition, and off-line analysis, we used a NI System (National Instruments, Austin, TX, USA) and custom-written MATLAB software (The MathWorks Inc., Natick, MA, USA). Optically evoked ABRs (oABRs) and acoustically evoked ABRs (aABRs) were recorded by needle electrodes underneath the pinna, on the vertex, and on the back near the legs. The difference potential between vertex and mastoid subdermal needles was amplified using a custom-designed amplifier, sampled at a rate of 50 kHz (National Instruments, Austin, TX, USA) and custom-written MATLAB software (The MathWorks Inc., Natick, MA, USA). Spikes were detected using an EPC-10 amplifier and spike probability was determined by counting the number of spikes observed during 200 Hz trains. A low-pass filter (300–3000 Hz) and averaged waveforms were collected for each presentation (for oABRs and aABRs, respectively). Thresholds were determined by visual inspection as the minimum sound or light intensity that elicited a reproducible response waveform in the recorded traces.

Juxtacellular recordings from single putative SGNs. For auditory nerve recordings15, a glass microelectrode (~25 MΩ) was advanced through the posterior end of the anteroventral cochlear nucleus, aiming toward the internal auditory meatus. Using an InChow microdrive (EXFO Bionics Ltd, Iaqi, MA, USA) and custom-written MATLAB software (The MathWorks Inc., Natick, MA, USA), extracellular action potentials were amplified using an ELC-03XS amplifier (NPI Electronics, Tamm, Germany), filtered (band pass, 300–3000 Hz), and digitized (TDT System 3) using custom-written MATLAB (Mathworks) software. Data were further analyzed and prepared for display off-line using custom-written Python (Python Software Foundation, Delaware, USA) and Matlab software. Once light-responsive fibers were encountered, stimulation was performed by means of 400 or 900 mW light-pulse trains at varying stimulation rates, leaving 100 ms inter-train recovery over 20 repetitions. Responses within the first 400 ms were then used for analyses. Only recordings for which the fibers generate at least five spikes per light-pulse train, and no responses across the 200 ms after the last light-pulse tested on each fiber were included. Phase-locking was quantified using the vector strength33 and its significance tested with the Rayleigh test. If L > 1.38, the null hypothesis was rejected at the 0.001 significance level57; insignificant VS were set to 0. The spike jitter, defined as the standard deviation of spike latency in one period of stimulation, was calculated using a time window equal to the stimulation period. The hazard function of the temporal jitter was evaluated for each stimulation rate by simulating Poisson spike trains at discharge rates from 10 to 300 spikes/s. The spike probability is the ratio between the number of spikes and the number of light-pulses. The temporal jitter is the standard deviation of spike latency across trials.

Data analysis. The data were analyzed using Matlab (The MathWorks Inc., Natick, MA, USA), Excel, Igor Pro 6 (Wavemetrics, Portland, OR, USA), Origin 9.0 (OriginLab Inc., Northampton, MA, USA), and GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Averages were expressed as mean ± s.e.m. or mean ± s.d., as specified. References to data in the main text were expressed as mean ± s.e.m. For statistical comparison between two groups, data sets were tested for normal distribution (Shapiro–Wilk test for normally distributed data and/or variance was unequal between samples. For evaluation of multiple groups, statistical significance was calculated by using one-way ANOVA test followed by Tukey’s test for normally distributed data (equality of variances tested with the Brown–Forsythe test) or one-way Kruskal–Wallis test followed by Dunn’s test for non-normally distributed data.

Data availability. The data that support the findings of this study and code used for analysis are available from the corresponding author upon reasonable request.
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H. (Fig. 6 and Fig. S11) performed research and analyzed data; J.J. prepared the AAVs; T. Ma., D.L.M., T.Mo. and E.B with contributions from V.S. and J.J.L. wrote paper.

**Additional information**

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