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Multichannel optogenetic stimulation of the auditory pathway using microfabricated LED cochlear implants in rodents

Daniel Keppeler^{1,2,*}, Michael Schwaerzle^{3,4,*}, Tamas Harczos^{1,5,*}, Lukasz Jablonski^{1,5}, Alexander Dieter^{1,2}, Bettina Wolf^{1,5}, Suleman Ayub^{3,4}, Christian Vogl¹, Christian Wrobel^{1,6}, Gerhard Hoch^{1,5}, Khaled Abdellatif^{1,5}, Marcus Jeschke^{1,5}, Vladan Rankovic^{1,5}, Oliver Paul^{3,4}, Patrick Ruther^{3,4,#}, Tobias Moser^{1,2,5,7,8,#}

¹Auditory Neuroscience and InnerEarLab, University Medical Center Göttingen, 37099 Göttingen, Germany

²Göttingen Graduate School for Neurosciences and Molecular Biosciences, University of Göttingen, 37075 Göttingen, Germany

³University of Freiburg, Department of Microsystems Engineering (IMTEK), 79110 Freiburg, Germany

⁴Cluster of Excellence BrainLinks-BrainTools, University of Freiburg, 79110 Freiburg, Germany

⁵Auditory Neuroscience and Optogenetics Laboratory, German Primate Center, Kellnerweg 4, 37077 Göttingen, Germany

⁶Department of Otorhinolaryngology, Head and Neck Surgery, University Medical Center Göttingen, 37099 Göttingen, Germany

⁷Collaborative Research Center 889, University of Göttingen, 37075 Göttingen, Germany

⁸Multiscale Bioimaging Cluster of Excellence, University Medical Center Göttingen, 37075 Göttingen, Germany

Abstract

When hearing fails, electrical cochlear implants (eCIs) provide the brain with auditory information. One important bottleneck of CIs is the poor spectral selectivity that results from the wide current spread from each of the electrode contacts. Optical CIs (oCIs) promise to make better use of the tonotopic order of spiral ganglion neurons (SGNs) inside the cochlea by

#Corresponding author. ruther@imtek.de (P.R.); tmoser@gwdg.de (T.M.).

*Equal contribution

Author contributions

The study was conceived and steered by T.M. and P.R. D.K. performed experiments, analyzed data. M.S. generated, optimized and in vitro characterized the LED-arrays under the supervision of P.R. and O.P. with contributions from S.A., T.H., L.J., G.H. and K.A. characterized the probes before and after physiology experiments, established driver hard- and software (including sound processor hardware) and the behavioral analysis platform, and interfaced them in collaboration with A.D., D.K. and B.W.. T.H. and L.J. developed firmware for sound processor under direction of T.M.. A.D. performed experiments together with D.K. and support of M.J.. B.W., A.D. and L.J. performed the behavioral experiments and their analysis. C.W. contributed to oABR recordings and analysis. V.R. prepared the AAVs used in the study and performed postnatal injections in rats and gerbils. A.D. performed ICC recordings and analysis. O.P. supported M.S. in the development of the thermal model. T.M., P.R., D.K., M.S. and A.D. prepared the manuscript. All authors contributed to manuscript preparation and reviewed the manuscript.

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spatially confined stimulation. Here we established multichannel oCIs based on light-emitting diode (LED) arrays and used them for optical stimulation of channelrhodopsin (ChR)-expressing SGNs in rodents. Power-efficient blue LED chips were integrated onto microfabricated 15- μm -thin polyimide-based carriers comprising interconnecting lines to address individual LEDs by a stationary or mobile driver circuitry. We extensively characterized the optoelectronic, thermal, and mechanical properties of the oCIs and demonstrated stability over weeks in vitro. We then implanted the oCIs into ChR-expressing rats and gerbils and characterized multichannel optogenetic SGN stimulation by electrophysiological and behavioral experiments. Improved spectral selectivity was directly demonstrated by recordings from the auditory midbrain. Longterm experiments in deafened ChR-expressing rats and in non-treated control animals demonstrated specificity of optogenetic stimulation. Behavioral studies on animals carrying a wireless oCI sound processor revealed auditory percepts. In conclusion, the study demonstrates hearing restoration with improved spectral selectivity by a LED-based multichannel oCI system.

Introduction

Hearing impairment (HI) is the most common sensory deficit and about 5% of the world's population suffers from disabling HI (1). Despite the dramatic clinical need and major research activities, a causal therapy is not yet available for the most common form: sensorineural HI (2–4). Hence, once HI has reached a severe state, hearing aids and CIs will remain key means for partial restoration of hearing. Electrical CIs (eCIs) use 12–24 electrode contacts covering a frequency range of about 100 Hz to 10 kHz (5) and restore speech recognition in the quiet to the majority of the 0.7 million CI-users. The spread of current from each electrode contact (6) leads to channel crosstalk (7) and diminishes the number of independent stimulation channels (8, 9).

Optogenetic stimulation of the cochlea is a promising approach to increase the spectral selectivity of CI coding because light can be conveniently confined in space. Indeed, recordings from the tonotopically layered central nucleus of the inferior colliculus (ICC) of rodents revealed a lower cochlear spread of excitation for optogenetic stimulation with single optical fibers than with electrical stimulation (10, 11). However, much remains to be done to develop optical stimulation as an alternative operating mode in clinical CIs (12). Next to establishing efficient and safe virus-mediated expression of appropriate opsins in auditory neurons (10, 13–16), a multichannel oCI system needs to be developed. Generally, active and passive oCIs can be considered (12). Waveguide arrays would passively propagate light from sources outside the cochlea, whereas active optoelectronic probes would convert current to light in encapsulated microscale emitters inside the cochlea. Interesting candidate emitters for blue optogenetic applications at the systems level are gallium nitride (GaN)-based LEDs (12, 17, 18). The LEDs can be integrated on flexible substrates using various approaches (19–22). Thus far, light sources used for optogenetic stimulation of SGNs have been individual GaN-based LEDs and optical fibers coupled to external lasers. LEDs or fibers were either placed onto a cochleostomy or into the cochlear scala tympani via the round window or cochleostomies (10, 11, 13–16). Single-channel optical stimulation has been an important step towards establishing the optogenetic control of the auditory pathway.

However, developing oCIs for auditory research and future clinical translation requires the generation of multichannel devices.

We report the design, fabrication and characterization of an LED-based multichannel optical cochlear implant system for optogenetic hearing restoration in rodents. Integrating highly power-efficient, commercially available GaN-based LED chips ($220 \times 270 \times 50 \mu\text{m}$) on a polyimide carrier, we studied electrical, optical, and thermal properties in vitro and optimized the electrical passivation and mechanical properties as required for chronic application in the rodent cochlea. A low-weight, wireless sound processor—a miniaturized stimulator—was devised and head-mounted on rats to drive the LED array in behavioral experiments. Using transgenic rats expressing ChR2-Venus under the pan-neuronal promoter Thy1.2 (23) or early postnatal injection of adeno-associated virus carrying the ChR2-mutant CatCh (24) into the rat or gerbil cochlea, we characterized oCI insertion and stimulation of the rodent auditory nerve by electrophysiological and behavioral analysis. A conference report on the technical development of the LED arrays has been presented (25). This work demonstrates the feasibility of improved spectral selectivity of SGN stimulation by an LED-based multichannel oCI system.

Results

Design, fabrication, assembly, and passivation of LED-based optical cochlear implants

To design LED-arrays for oCIs with appropriate dimensions and mechanical properties, we first performed X-ray tomography of the rat cochlea (26) yielding estimates of the cross-section of the scala tympani and its curvature (Fig. 1, A and B). The cross-section has a width of about $1000 \mu\text{m}$ at the cochlear base, drops to about $300 \mu\text{m}$ in the mid-cochlea, and then levels off. Likewise, the radius of curvature starts large (about $1400 \mu\text{m}$) and declines to about $400 \mu\text{m}$ in the apex. Hence, we engineered slim and flexible arrays not to exceed $300 \mu\text{m}$ in diameter after encapsulation and for mechanical and functional compatibility, with a bending radius of about $500 \mu\text{m}$.

The design of the oCIs based on a polyimide (PI) substrate and commercially available LED chips emitting at 457 nm is illustrated in Fig. 1C. It consists of a slender implantable section (width: $240 \mu\text{m}$ or $283 \mu\text{m}$) with a linear array of LEDs at its tip wired to the interface section which is designed for 17-pad zero insertion force (ZIF) connectors. The central contact pads of the ZIF connector section and the respective wiring lines (a broader common p-contact line and ten n-contact lines, one for each LED) address individual LEDs for their independent control. The linear array of ten LEDs at the oCI tip was designed with a pitch of $500 \mu\text{m}$ or $350 \mu\text{m}$. In addition, an array with four LEDs containing a temperature sensor based on a platinum meander integrated beneath the first LED chip was established (Fig. 1D). The four-wire interconnection of this meander enables a precise resistance measurement and thus allows monitoring a potential temperature increase inside the implant while operating the LED chips.

To fabricate the oCIs (see Methods) we first generated the PI-based carrier on a silicon (Si) handle wafer (fig. S1). The microfabrication steps included: the subsequent spin coating and curing of three PI layers patterned by reactive ion etching (RIE); the sputter deposition,

lift-off structuring, and electroplating of metal layers used for the Pt-based integrated temperature sensor (first metallization); and LED wiring (second metallization; thickness 1 μm) and contact pads (third metallization; thickness 5 μm). Chronic oCIs were comprised of only two PI layers and two electroplated metallizations for LED wiring and contact pads, whereas the integration of a Pt-based temperature sensor was omitted from this design variant.

Functionality tests were performed before and after device passivation by sequentially addressing all LEDs (LED 1 – LED 10, Fig. 1G). In addition, motivated by the snail-like structure of the cochlea, we tested the mechanical oCI functionality by inserting the optical implant into 300- μm -wide channels with different outer radii made in polymethylmethacrylat (PMMA). We determined the minimal outer radius, which differed depending on the LED pitch and the thickness of the silicone encapsulation. Figure 1H demonstrates the function of all ten LEDs of an oCI (LED pitch of 350 μm , mold-encapsulated to an outer diameter of 300 μm) bent to an outer radius of 1 mm. oCIs with an LED pitch of 500 μm and a silicone thickness of approximately 50 μm could be bent to a radius of only 500 μm while remaining functional.

Electrical, optical and thermal characterization of LED-based optical cochlear implants in vitro

The electrical and optical characterization of the oCIs was performed by measuring the LED voltage V (Fig. 2A) and radiant flux Φ (Fig. 2B) as a function of the LED driving current I_{LED} which was varied between 0 to 45 mA and pulsed at a duty cycle (DC) and a frequency of 10% and 10 kHz, respectively, resulting in pulses of duration $t_p=10\text{ }\mu\text{s}$. The threshold voltage of the applied LEDs (extracted from Fig. 2A) was $V_{th} = 2.6\text{ V}$ which is comparable to the value of 2.7 V given in the LED datasheet (27). The slopes of the I - V -curve are determined by the LED characteristics and the line resistance of the respective LED wiring, which likely explains the small differences among the various LEDs. On average a voltage of $4.32\pm0.04\text{ V}$ was measured at $I_{LED} = 30\text{ mA}$. The measurements of the optical radiant flux Φ as a function of I_{LED} (Fig. 2B) were performed using a calibrated integrating sphere and a spectrometer. The LEDs of this representative probe showed the same behavior and provided a time averaged radiant flux of $1.41\pm0.05\text{ mW}$ at $I_{LED} = 30\text{ mA}$ (10% DC and 10 kHz). This corresponds to a radiant flux of 14 mW during the 10- μs -long light pulses. Taking the LED footprint of $220\times270\text{ }\mu\text{m}^2$ into account, 236 mW/mm^2 were emitted during those current pulses. Time averaged radiant emittances of 10, 15, and 20 mW/mm^2 relevant for optogenetic experiments (10) were consequently emitted at $I_{LED} = 1.25, 1.61,$ and 2.27 mA , respectively, applying a DC of 10%. Spectral measurements of assembled and passivated LEDs showed light emission with an averaged center wavelength of $453.8\pm1.1\text{ nm}$ (Fig. 2C).

Since the oCIs are active devices with limited optoelectronic conversion efficiency, we set out to characterize heat production and dissipation using the integrated Pt meander to determine the temperature increase inside the probe, close to an operating LED chip. Sensor calibration and the measurement setup are described in detail in the Supplementary Material. Based on a set of transient temperature measurements performed by applying different LED

currents I_{LED} and switching on the LED for a duration t_{on} , the thermal time constant of the system was determined to be 11.8 ms. The experiments were performed with the oCI sandwiched between layers of agarose gel kept at 37°C aimed to mimic the thermal properties of the fluid-filled cochlear scala tympani. The experiments allowed extraction of thermal relaxation functions modeling the temperature response of the probe to the LED operation and prediction of the maximum temperature increases inside the oCIs for given I_{LED} and on-times t_{on} of the LEDs. As expected, the modeled temperature evolution under the operated LED in response to $t_{on} = 4$ ms increased with I_{LED} (Fig. 2D). Correspondingly, for a given I_{LED} at 100% DC (38.9 mA, maximal I_{LED} used in physiology resulting in $\Phi = 18.1$ mW) the maximum temperature T_{max} rose with pulse durations t_{on} between 0 and 10 ms (Fig. 2E). Figure 2F shows T_{max} as a function of Φ for $t_{on} = 1, 2$, and 4 ms. Hence, to operate the oCI at a given stimulation rate below a certain temperature, I_{LED} and thus radiant flux or pulse duration should be limited. Because the temperature is measured within the oCI close to the heat-generating LED, it overestimates the temperature increase in the surrounding tissue. In addition, the protocol used for the worst-case estimation ($I_{LED} = 38.9$ mA at 100% DC and $t_{on} = 10$ ms) exceeds what seems feasible for in vivo stimulation.

Next, we simulated temperature profiles inside and outside the oCI while immersed in an aqueous medium mimicking the thermal coupling to the perilymph fluid inside scala tympani (Fig. 2, fig. S3 and table S1). The simulated cross-sectional temperature profile for the worst-case scenario ($I_{LED} = 38.9$ mA at 100% DC and $t_{on} = 10$ ms) is shown in Fig. 2G. Due to the short distances and the high thermal conductivity of the LED substrate [silicon carbide (SiC)], simulated temperature increases of +37.2 K, +17.7 K, and +13 K were obtained at the top, side, and bottom surfaces of the silicone-encapsulated oCI, respectively (Fig. 2H). Outside the oCI, at a distance of approximately 100 μ m (a realistic position relative to the oCI of, e.g., the medial cochlear wall) the temperature increase was below 1 K even for $t_{on} = 10$ ms (Fig. 2H).

Longevity tests were performed on oCIs which had been processed with different underfill and/or encapsulation materials in the absence of the final silicone encapsulation for scrutinizing the LED-proximal encapsulation. In saline solution, the integrated LEDs were reverse biased by a non-permissive voltage of -5 V between their n- and p-lines measuring the leakage current I_{leak} (for details see Supplementary Material – Experimental characterization). I_{leak} values of three representative oCI samples show a dependence of longevity on the underfill and encapsulation procedure (Fig. 2I). The oCI underfilled using the solvent-containing fluoropolymer Cytop only, failed within 8 hours as evident by the increasing I_{leak} (Fig. 2I). In contrast, the oCI underfilled by solvent-free epoxy, with a surface activation by an argon plasma before and after the underfill, survived for more than 30 days. This indicates an advantage of using a solvent-free underfill, as further detailed in fig. S2, C and D. Finally, combining the repeated plasma treatment with epoxy underfill and Cytop encapsulation further increased the longevity of the oCI to more than 200 days. These longevity results are summarized in Fig. 2J for the oCI samples characterized to date. We suggest that the additional silicone encapsulation as used for the in vivo experiments further supports mechanical stability and increases longevity enabling chronic in vivo oCI studies.

Optogenetic activation by multichannel oCIs: basic characterization

Next, we turned to transgenic rats broadly expressing ChR2 under the Thy1.2 promotor [ChR2-Venus rats (23)] to test the feasibility of multichannel LED-based oCI activation of the auditory pathway by recording optogenetically evoked brainstem responses (oABR) in acute experiments. The oCIs were connected via their ZIF connector interface (Fig. 3A) to a stationary microprocessor-controlled current source driver circuitry. Prior to each experiment we measured the emitted light power per LED as a function of I_{LED} with an integrating sphere and probed the function and electrical integrity of the oCIs in saline. Then, we performed a posterior tympanotomy (Fig. 3B), inserted the oCI probe through the round window, and gently advanced it into the scala tympani. In a few cases we inserted the oCI through a cochleostomy in the middle cochlear turn. After recordings of optogenetically evoked auditory brainstem responses (oABR) were performed, the position of the implant was validated by X-ray phase-contrast microscopy as described before (26). With round window insertion and without forcing, the oCIs usually covered the basal turn of scala tympani (Fig. 3C, Movie S1, Movie S2). We then compared supra-threshold auditory brainstem responses (ABR) evoked by acoustic clicks (aABR, Fig. 3D, upper curve), 4-ms-long laser light pulses (oABR, Fig. 3D, middle curve, 473 nm, 6.6 mW delivered via a 200 μ m optical fiber inserted into the scala tympani via the round window), and oCI stimulation with the LED-based implant (oABR, front LED, 457 nm, 6.8 mW, Fig. 3D, lower curve). The oABRs (asterisks mark the first positive peak, p1) elicited by the LED-stimulation were preceded and followed by an electrical artifact that was variable in size. Except for these I_{LED} -related artifacts, the oABRs elicited by laser and LED stimulation at comparable light intensities were similar. Both were also comparable in amplitude to aABR, but shorter in latency and showed less prominent later ABR peaks. The oABR strictly depended on the placement of the LED within the scala tympani (Fig. 3E): when placed in the bulla (middle ear), no oABR could be elicited. Moreover, oABRs vanished after euthanizing the animal, despite intracochlear stimulation (Fig. 3E). We never observed oABRs in ChR2-negative rats, whereas oABRs were found in a ChR2-transgenic rat after deafening using intracochlear injection of the ototoxic drug kanamycin (Fig. 3E, figs. S4 and S5). This demonstrates the specificity of the optogenetic stimulation and rules out optoacoustic (30), optothermal (31), or direct electrical stimulation. Individual LEDs of the optical implant evoked slightly different oABR waveforms (Fig. 3F). Although the oCI contained 10 LED chips, we were typically able to place only up to 5 of them inside the scala tympani with gentle insertion via the round window, resulting in stimulation of the basal turn. X-ray tomography indicated that this was likely because the oCI tip did not glide further into the scala tympani due to contact with the lateral wall.

Optogenetic activation by multichannel oCIs: reproducibility, reliability and safety

We then characterized the reproducibility and reliability of the multichannel oCIs in acute experiments. First, we compared the oABRs elicited by LED 1 of five different oCIs inserted into the scala tympani of the same rat via cochleostomy for most cautious insertion (Fig. 4A). The p1-morphology of the oABRs was qualitatively conserved with some variability of amplitude and latency (Fig. 4, A and B). The electrical artifacts varied among oCI insertions. The p1-amplitude grew over more than one order of magnitude of radiant flux and did not show evidence for saturation at high radiant fluxes. At high radiant flux (18 mW), stimuli as

short as 100 μ s were sufficient to elicit oABRs, which grew in amplitude with duration up to 2 ms and then leveled off (Fig. 4C). The p1-amplitude decreased with increasing stimulus rate, but remained detectable up to 200 Hz (Fig. 4D).

Next, we compared oABRs evoked by laser light (optical fiber implanted via cochleostomy) to those driven by an oCI (implanted via cochleostomy) in 5 different animals (Figs. 4, E to H). We first studied the response to the laser and then inserted an oCI, which showed only small electrical artifacts. We observed a substantial variability in oABRs to LED-1-stimulation among the animals. The oABRs evoked by oCI in the five rats were comparable in amplitude to those elicited by laser pulses of the same radiant flux (Fig. 4F). The dependence of the oCI-evoked oABRs on pulse duration (Fig. 4G) was comparable to those obtained with the other oCIs (Fig. 4C), but differed from those driven by the laser with higher radiant flux, with which we observed a tendency of the oABRs to decline beyond durations of 2 ms (Fig. 4G). The rate-dependent p1-amplitude decline seemed shallower for LED-stimulation than for laser stimulation (Fig. 4H).

Finally, we measured the temperature increase inside the cochlea when operating oCI probes in the scala tympani and running a repetitive and parallel optical stimulation. These experiments used the oCI probe design employed in chronic experiments that did not include the Pt meander temperature sensor to reduce complexity. Instead we inserted the tip of a commercial temperature sensor into the scala tympani via the round window, while implanting the oCI via a basal cochleostomy (fig. S6). In two naïve rats, we did not encounter a measurable rise of temperature upon intracochlear optical stimulation using parallel activation of 6 LEDs (stimulation with 1 ms pulses for 30min and subsequently with 4 ms pulses for 17 min at maximum optical power, fig. S6).

Optogenetic activation by multichannel oCIs: spectral selectivity

The development of the oCI is motivated by improved spatial confinement of artificial SGN stimulation which promises better spectral selectivity and hence a greater number of independent stimulation channels along the cochlear tonotopic axis. LEDs are Lambertian emitters with wide-angle beam profiles. Therefore, and because of the relatively large size of the LEDs ($220 \times 270 \mu\text{m}^2$) used here, this study testing LEDs in the relatively small cochlea of rodents will likely provide a lower estimate of the spectral selectivity achievable with future clinical oCI implementations (32). To estimate the spectral selectivity of optogenetic SGN-stimulation by LED-based multi-channel oCIs, we used two acute experimental approaches.

First, we performed oABR recordings aiming to study potential additive effects of LEDs recruited in addition to a single LED (fig. S7). The reasoning was that, if indeed each LED activates different SGN subpopulations, the oABR amplitude should grow when recruiting additional, more basal LEDs that would activate further SGNs. Figure S7 shows an exemplary experiment in which we subsequently recruited additional LEDs and studied the effects on the oABR amplitude for different radiant flux, pulse durations, and stimulation rates. Additivity was strongest when activating the second LED in addition to LED 1 and a lower gain in amplitude was found when recruiting further LEDs. Additivity of stimulation

by multiple LEDs indicates that the spread of excitation from the LEDs is limited at least at low stimulus intensity such that additional LEDs could drive further SGNs.

Second, we performed electrophysiological recordings of multi-unit activity from the central nucleus of the inferior colliculus (ICC) using linear 32-channel multi-electrode arrays. We chose the Mongolian gerbil because it has a larger cochlea than the rat and hence allowed for more ideal placement of the oCIs. Since both the spiral ganglion and the ICC are characterized by a remarkably conserved tonotopic organization, the cochlear spread of excitation can be inferred from the neural activation observed in the ICC. oCIs were inserted into the cochlea via a mid-turn cochleostomy to excite the center of the spiral ganglion and thus estimate the spread of excitation in the center of the ICC rather than at its dorsal or ventral edges, where neural activation would reach the borders of the electrode array and thus underestimate the spread of excitation. Measurements were performed in gerbils 5–6 months after early postnatal (postnatal day 7) injection of AAV-PhP.B carrying the ChR2 mutant CatCh under the synapsin promoter into the left cochlea. CatCh expression in SGNs was demonstrated by posthoc immunohistochemistry (fig. S8). To interpret neural activation in response to oCI stimulation and compare data across animals, electrode positioning along the tonotopic axis of the ICC was physiologically confirmed by free-field acoustic stimulation using pure tones of varying frequency and intensity. After mapping acoustic responses in the ICC, oCIs were implanted and individual LEDs were driven at different intensities. Subsequently, oCIs were removed and clinical-style, 4-channel eCIs (provided by MED-EL) were implanted to drive the auditory nerve electrically and compare the spread of excitation in the same animal. ICC activation by artificial SGN stimulation was evaluated based on peri-stimulus time histograms (PSTHs) in response to maximum stimulus parameters (individual LEDs driven at 30 mA, 1 ms, ~8.65 mW radiant flux as measured by LED calibration before the experiment, mono- and bipolar electrical stimulation at 400 μ A; Fig. 5, A to C). Neural responses were found to occur 4.5–23.75 ms after optogenetic and 2.5–14.75/2.5–13 ms after mono- and bipolar electrical stimulus onset, respectively. To avoid stimulation artifacts, a period of ~2 ms was linearly interpolated in the raw data traces, starting 0.5 ms before stimulus onset. Based on PSTHs, response windows for potential multi-unit activity were set to 2–25 ms for optogenetic and 2–15 ms for electrical stimulation. During these time windows, multi-unit activity in response to a given stimulus was sorted in a two-dimensional matrix according to the corresponding recording site and stimulus intensity. Subsequently, a cumulative discrimination index (d') was calculated based on multi-unit firing rates in response to increasing stimulation intensities, starting with a zero-intensity condition [no stimulation, baseline (11, 33, 34)]. The cumulative d' quantifies the change in response strength as standard deviations from baseline firing: d' of one is equal to a rise in firing rates by one standard deviation. To estimate the patterns of ICC activation, spatial tuning curves (STCs) were constructed based on iso-contour-lines at integer d' values (Fig. 5, D to F) for optogenetic as well as mono- and bipolar electrical stimulation.

The recording site with the lowest threshold was defined as the best electrode, and the spatial extent of neural activation in the ICC was defined as the range of recording sites covered by electrodes that recorded responsive multi-units ($d' \geq 1$) at the stimulus intensity, which corresponds to a fixed d' value at the best electrode. This activity-based quantification

allows for an estimation of neural excitation independent of the nature and absolute intensity of a stimulus and thus enables the comparison of spectral selectivity upon optogenetic and electric stimulation at comparable activation strengths. Using tonotopic slopes (Fig. 5G), which have been calculated for each animal based on the acoustic mapping of the ICC, the spatial spread of excitation in the ICC could then be transformed into spectral spread of excitation (Fig. 5H).

We observed that the spread of cochlear excitation upon optogenetic stimulation using individual LEDs was comparable to mono- and bipolar electrical stimulation at low stimulus intensities but outperformed both modes of electrical stimulation at activation strengths above a d' of 2, confirming the improved spectral selectivity of optogenetic stimulation as compared to electrical stimulation. At a d' of 3, optogenetic stimulation was 1.7-fold more selective than both mono- and bipolar electrical stimulation (mean spread of excitation: 4.1/6.9/6.9 octaves for optogenetic/mono/bipolar stimulation, respectively). No significant differences could be observed between mono- and bipolar electrical stimulation ($p = 0.57/0.84/0.91/1$ at a d' of 1.5/2/25/3, respectively, repeated-measures ANOVA and post-hoc pairwise comparison). The cochlear spread of excitation upon LED stimulation exceeded the one reported for stimulation with an optical fiber at low stimulus intensities, but was comparable at stimulation strengths of a $d' > 2$ (dashed line in Fig 5H).

Behavioral analysis of optogenetic activation of the auditory pathway by multichannel oCI system

A multichannel oCI system for chronic application requires long-term stable intracochlear emitter arrays as well as appropriate sound processors. To minimize the risk of device failure, we simplified the design to use two PI layers and two electroplated metallizations for LED wiring and contact pads. For operation of the oCI in behavioral experiments (negative reinforcement shuttle box learning paradigm), we developed a low-weight (8 g), complete multichannel oCI sound processor (Fig. 6). The detailed technological description and characterization will be described in a separate manuscript (Jablonski et al., bioRxiv 2020, DOI: 10.1101/2020.05.25.114868). In brief, the oCI sound processor consists of a microelectromechanical systems (MEMS) microphone (Knowles SPH0641LM4H-1) with digital pulse density modulation (PDM) interface, an ARM Cortex M4f-based digital signal processor (DSC) with embedded 2.4 GHz radio module (Nordic Semiconductor nRF52832), an LED driver (Texas Instruments TLC5923), and a battery. All elements were integrated onto two stacked, round printed circuit boards (PCBs) of 2 cm in diameter and connected to the head-mounted pin connector interfacing to the ZIF-connector of the oCI (Fig. 6, B and C). The whole assembly was housed in a light (6.5 g) plastic case consisting of a base mounted to the animal's skull by dental acrylic and two metal anchors, as well as a screwable cap. From the pin connector, a polyimide-embedded cable of the chronic oCI was funneled underneath the skin towards posterior tympanotomy to drive the intracochlear LED array.

Due to greater physical strength, we used mature rats 5–6 months after early postnatal left cochlear injection of AAV-PHP.B carrying the ChR2 mutant CatCh under the synapsin promoter (Fig. 7). The chronic oCI was implanted after acoustic training of avoidance

behavior in the shuttle box negative reinforcement learning paradigm (14) in rats that had undergone AAV-mediated optogenetic manipulation ($n = 2$) and naïve rats ($n = 2$). The AAV-transduced rats were deafened by bilateral cochlear injection of kanamycin during the oCI surgery (Fig. 7A) whereas in naïve rats hearing ability in the right ear was preserved (fig. S9). Functional CatCh expression and optogenetic activation of the auditory pathway were confirmed by recording oABR in response to fiber-based single channel optical stimulation of the cochlea (prior to the insertion of the oCI, Fig. 7B) and deafness confirmed by lack of acoustic ABR (aABR, Fig. 7C). Animals were allowed to recover for one week. We then fit the oCI system with increasing stimulation strength and adapted the rats to intermittent burst stimulation of all functional LEDs on the array in their home cage.

In the subsequent weeks, the rats were tested for perception of LED-based oCI stimulation of the auditory nerve in the shuttle box (Fig. 7D). Upon wireless triggering of intermittent burst stimulation of all functional LEDs in the array, the CatCh-expressing rats performed the task with a hit rate of about 80% (Fig. 7E, Movie S3) whereas the non-injected control rats continued responding to acoustic stimulation but not to optical stimulation in the cochlea (fig. S9). The lack of response in the control rats excludes potential optoacoustic, optothermal, or electrical SGN activation by the oCI in the experimental conditions. The optically-cued avoidance behavior of CatCh-expressing rats, and hence the functionality of our oCI system, was stable over more than 1 month. In a third setup, we tested the operation of the complete oCI system in response to acoustic stimulation. The deaf CatCh-expressing rats showed avoidance behavior when the sound processor was powered on, but could not use the acoustic signal as a cue when the sound processor was powered off in a fourth experimental setup (Fig. 7F). This demonstrates the basic functionality of the oCI system and behaviorally confirms the deafness of the rats.

Discussion

Here we demonstrate the design, fabrication, characterization, and application of LED-based multichannel optical cochlear implants in rodents. The design and fabrication of these oCIs was guided by the diameter of the scala tympani of the rat cochlea ($>300\text{ }\mu\text{m}$), as estimated from X-ray tomography, and a specified minimal bending radius (1 mm). This placed constraints on the design of oCIs for rodents, also restricting the number of integrated LED chips, which were individually addressed as is the case of electrodes in current clinical eCIs. As the applied LED chips require currents in the mA-range for light emission that activates optogenetically-modified SGNs, the line width and thickness of the LED feed lines were optimized to minimize resistive losses. The LED dimensions of $270\times220\times50\text{ }\mu\text{m}^3$ were small enough for implantation into the rodent cochlea and large enough for LED assembly using flip-chip bonding. The LED size and pitch limited the bending radii of the oCI. Reducing probe dimensions and bending radii can be achieved by the integration of thin-film μLEDs , as recently demonstrated (20, 22). The optical characterization of the radiant flux indicated that sufficient optical power can be generated for optogenetic activation of SGNs, which was directly confirmed by biological experiments. The temperature modeling, based on measurements with passivated oCIs in contact with agarose gel, enabled the determination of the implant temperature in a worst-case scenario. The thermal simulation shows that the temperature increase -- even for the highest LED currents and longest

stimulation pulses -- never exceed 1 K at a distance of $\sim 100 \mu\text{m}$ which is below the assumed distance of the oCI to the SGNs. These estimates are comparable to those recently obtained for LED-stimulation of the mouse heart (35) and seem acceptable according to the ISO 14708-1 standard for implantable medical devices limiting the temperature increase to 2 K. In fact, our first in vivo measurements did not show a noticeable rise of cochlear temperature upon prolonged (47 min at 10 Hz, 1 and 4 ms) and parallel operation of 6 LEDs at maximum optical power.

The LED-based multichannel oCIs could be readily inserted into the basal high frequency turn of the rat scala tympani via the round window or cochleostomy. X-ray tomography indicated that the oCI tip engaged with the lateral wall, likely owing to limited flexibility and, hence, bending. Increasing the flexibility by smaller LEDs and/or larger pitch, cochleostomy at optimized position, as well as shaping and softening the oCI tip will likely improve the insertion. The LED-based multichannel oCIs elicited oABRs comparable to those evoked by laser-pulses but flanked by electrical artifacts. Since no leakage currents were observed, we assume that the artifacts were of capacitive origin, which calls for a better electrical shielding of the oCI supply lines. The oCI-elicited oABRs were highly specific as they were absent when (i) the stimulating LED chip was outside the scala tympani, (ii) the animal was euthanized, and (iii) wild-type rats lacking ChR2-expression in SGNs were stimulated. Moreover, the oABRs could be elicited in ears that had been ototoxically deafened. These controls rule out optoacoustic or electrical stimulation to contribute to SGN activation.

Sequential insertion of several oCIs into a given rat cochlea, as well as the subsequent insertion of one oCI in several rats, was possible and compatible with function, indicating general utility and sufficient mechanical and electrical stability of the oCI prototypes. Some variability of oABRs was observed with both experiments, however it seemed less pronounced than in our previous experiments in which oABRs were vastly different in response to the focused light of a power-LED (10). Unlike in our previous study on ChR2-mediated optogenetic stimulation of the auditory nerve, in which oABRs failed below 100 Hz (10), here we could demonstrate oABRs elicited by oCIs in ChR2-transgenic rats for stimulus rates as high as 200 Hz. We cannot fully explain this discrepancy, however we speculate that it might relate to more specific activation of the auditory pathway in the present study.

The spectral selectivity of the LED-based multichannel oCIs was demonstrated by additivity of the oABR amplitudes elicited by neighboring LEDs and by recordings of ICC activity. Based on the latter, the spread of excitation with LED-based oCIs (1.9/2.4/3.4/4.1 octaves for a d' of 1.5/2/2.5/3) outperformed both mono- and bipolar eCI stimulation (1.6/3.6/6.5/6.9 and 2.2/4.2/6.2/6.9 octaves, respectively) and thus confirmed the hypothesis of increased spectral resolution of optogenetic sound encoding. However, LED-based optogenetic SGN stimulation did not yet reach the selectivity of pure tone acoustic stimulation in gerbils that did not undergo cochlear surgery [0.9/1.4/1.7/2.2 octaves; reported in a different study using identical experimental setup and analysis (11)]. Establishing and characterizing arrays with a higher density of smaller and highly efficient thin-film GaN μLEDs will be an important next step. Thin-film GaN μLEDs can reach power efficiencies of up to 50% (36) and are

good candidates as emitters for ‘active’ blue oCIs (12). Focusing by micro-lenses (37, 38) and/or ‘modiolus-hugging’ (39) in order to bring the oCI as close as possible to the medial wall of the cochlea and to let the LEDs directly face the SGN somas in Rosenthal’s canal can be expected to further reduce the spread of excitation.

Finally, LED-based multichannel oCIs were used in chronic behavioral studies. AAV-mediated expression of CatCh in rat SGNs was established and mediated potent sensory cues for avoidance behavior in the shuttle box paradigm. We implemented the complete oCI system, which allowed the deafened animals to execute the behavior in response to direct LED stimulation and, importantly, to acoustic stimulation which was turned into oCI stimulation by the custom-made sound processor. The low-weight system was well tolerated by the rats and can now serve in depth behavioral and electrophysiological studies in rats and other larger rodents and potentially cats and non-human primates.

Limitations of the present study include the large size of the LEDs and broad spread of light from each emitter, which make the present estimate of spectral selectivity of optogenetic stimulation represent a lower bound. Moreover, full insertion of the oCI was not regularly achieved which calls for optimizing the oCI design. Finally, the study only provides a first proof of concept of multichannel optogenetic stimulation and much remains to be done to prepare a clinical translation of the approach for improved hearing restoration with future oCIs. For instance, future studies should perform a behavioral analysis of spectral selectivity of oCI stimulation.

In conclusion, this study demonstrates the feasibility and improved spectral selectivity of optogenetic stimulation of the auditory pathway by LED-based multichannel oCI system. Further work is required to translate LED-based multichannel oCIs into a clinical application.

Materials and Methods

Study design

We aimed for design, fabrication, characterization, and application of LED-based multichannel optical cochlear implants in rodents for evaluating their potential of optogenetic hearing restoration. Rats and gerbils expressing channelrhodopsin in the spiral ganglion neurons of the cochlea were implanted with the microfabricated optical cochlear implants and further investigated with physiological, immunohistochemical, imaging and behavioral methods.

Microfabrication of the optical cochlear implant substrates

Figure S1 summarizes the microfabrication of the highly flexible triple layer polyimide (PI) substrates. Their total thickness (15 μm) results from three layers of PI and three metal layers. A first 5- μm -thick PI (UPIA-ST, UBE Europe GmbH) layer was spin-coated onto a silicon (Si) handle wafer and cured at 450°C for 3 h (fig. S1A). This was followed by the first metallization [platinum (Pt), 250 nm] which was sputter-deposited after applying an oxygen plasma activation of the PI surface at 80 W for 1 min. This first metal layer was lift-off patterned using an image reversal photoresist (AZ5214E, Merck KGaA). The second

PI layer was spin-coated to a thickness of 2.5 μm and patterned using a photoresist mask (AZ9260, Merck KGaA) and reactive ion etching (RIE) (fig. S1B). To improve the adhesion of this second PI layer to the PI substrate an oxygen plasma (80 W, 1 min) was again applied. The second metallization comprising the LED supply lines made use of a sputter-deposited seed layer (Pt/gold (Au)/titanium (Ti), 30/200/30 nm). The seed layer adhesion was improved by an argon (Ar) plasma treatment directly prior to the metal deposition. This was followed by deposition and patterning of a second photoresist mask, the removal of the upper 30-nm-thick Ti adhesion layer using 1% hydrofluoric acid (HF) and electroplating of Au to a thickness of 1 μm for the LED wiring (fig. S1C). After removal of the electroplating mask, the remaining Ti and Au seed layers around the electroplated structures were wet etched using 1% HF and potassium iodide/iodine (KI/I₂) solution, respectively, while the Pt layer was dry etched using an Ar plasma (fig. S1D). For the third PI layer with a thickness of 1 μm and the third metallization of the LED bond pads with a thickness of 5 μm we applied the same procedure as for the second layer level (fig. S1, E to G). The PI and thus probe patterning employed RIE using a 30- μm -thick AZ9260 photoresist as the masking layer (fig. S1H). Finally, the oCI substrates could be peeled off the wafer using tweezers (fig. S1I).

The sequential integration of the LED chips onto the PI substrates used flip-chip bonding which enabled the accurate and reliable assembly of individual LEDs to the electroplated bond pads by applying force, temperature, and ultrasonic agitation. The n- and p-pads of the LEDs are visible through the transparent LED substrate (Fig. 1, E and F). Polymeric encapsulation layers were manually applied after the LED assembly (fig. S2). The oCIs received a polymeric underfill of the LEDs (epoxy EPO-TEK 301-2) and were encapsulated in a thin fluoropolymer film (Cytop) as well as in silicone, all applied by a manual dispensing process (probes shown in Fig. 1, E and F) or using a precision-machined mold (probes shown in Fig. 1, G and H). The thicknesses of the fluoropolymer layer and underfill were in the low micrometer-range and defined by the height of the electroplated LED pads underneath the LEDs, while the silicone layer was about 30-60 μm in thickness.

In the case of the chronic oCIs, overall fabrication process was simplified using the following sequence of layers and process steps: Spin-coating of PI (5 μm), deposition and patterning of Pt layer, deposition of seed layer, electroplating of Au (1 μm , LED feed lines), removal of seed layer around the electroplated LED lines, spin-coating of second PI layer (5 μm), RIE of vias into the second PI layer, deposition and patterning of Pt (LED bonding pads and ZIF contact pads), deposition of seed layer, pads thickening using Au electroplating of bonding and contact pads (5 μm), removal of seed layer around the electroplated pads, RIE patterning of dual-layer PI stack to define the probe shape, and finally substrate peel-off.

Animals

Experiments were performed on adult wildtype and transgenic channelrhodopsin-2 rats [Thy1-ChR2-Venus, (23)] of either sex, as well as on wildtype Wistar rats and gerbils in which SGNs were transduced via intracochlear injection 6/7 days after birth of AAV-PHP.B carrying with the calcium translocating channelrhodopsin CatCh under the control of the human synapsin promoter. All experiments conformed to the local and national guidelines

for the care and use of laboratory animals in research and were approved by the local authorities of the State of Lower Saxony (LAVES).

ABR recordings

oCIs or a 200- μ m optical fiber coupled to a blue laser (473 nm, MLL-FN-473-100, 100 mW DPSS; Changchun New Industry Optoelectronics) were inserted into the cochlea via the round window (or via a cochleostomy, where indicated). The radiant flux of laser and oCIs were calibrated with a laser power meter (Gentec-EO Solo 2) and a calibrated integrating sphere, respectively. The LED-based optical probes were controlled via digital-to-analog (DA) outputs achieving pulse sequences with a resolution of 2 μ s driven with an operational amplifier. The light output was controlled by the voltage output of the the DA. ABRs were recorded by needle electrodes underneath the pinna and on the vertex, while an active shielding electrode on the neck was used to reduce noise. The differential potential between vertex and mastoid subdermal needles was amplified using a custom-designed amplifier (gain 10,000), sampled at a rate of 50 kHz (NI PCI-6229, National Instruments), and filtered off-line (0.3 kHz to 3 kHz Butterworth filter) for acoustically and optically evoked ABRs. Stimulus generation and presentation (acoustic and optic), and data acquisition were realized by a customized software in MATLAB (The MathWorks, Inc.) employing National Instruments cards in combination with custom-built hardware to amplify and attenuate audio signals. In case of acoustically evoked ABRs, clicks of 0.3 ms length were presented in an open near field via a single loudspeaker (Vifa, Avisoft Bioacoustics) placed 30 cm in front of the animal at the level of the animal's head. Sound pressure levels were calibrated with a 0.25-inch microphone (D 4039, Brüel & Kjaer GmbH) and measurement amplifier combination (2610; Brüel & Kjaer GmbH).

Deafening

After performing a posterior tympanotomy and accessing the cochlea, 2–3 μ l of kanamycin solution (100 mg/ml; Kanamysel; Selectavet) were injected into the left cochlea via the round window membrane prior to oCI insertion. To reduce the duration of the surgery of the chronically oCI implanted AAV-transduced rats, the same amount of kanamycin solution was injected into the right cochlea via a transtympanic approach.

Recording of Multi-Unit Activity (MUA) in the central nucleus of the inferior colliculus

Multi-neuronal clusters from the central nucleus of the inferior colliculus (ICC) contralateral to the injected ear were recorded with linear 32-channel silicon probes (50 μ m electrode spacing, 1–3 M Ω impedance at 1 kHz; Neuronexus), as described (11). Briefly, the ICC of stereotactically head-fixed gerbils was accessed by an incision on the animal's skull and a craniotomy was performed approximately 2 mm lateral and 0.5 mm caudal to Lambda using a dental drill. After removing the dura from the visual cortex, which in gerbils covers the inferior colliculus, the silicon probe was slowly inserted into the brain (~2 mm lateral to lambda on the mediolateral axis and as close as possible to the transverse sinus on the rostrocaudal axis) to a depth of ~3.3 mm using a micromanipulator (LN Junior 4RE; Luigs & Neumann). After a waiting period of one hour to allow for relaxation of neural tissue, an initial mapping of characteristic frequencies was performed. Based on neuronal responses the probe was further advanced or retracted in order to make ideal use of the tonotopic

axis of the ICC and enable comparable electrode positioning across animals. A metal wire (impedance: $< 1 \Omega$) on the cortical surface of the contralateral hemisphere served as a reference electrode. Multi-unit activity was amplified, filtered (0.1 Hz–9 kHz) and recorded (32 kHz sampling rate) using a Digital Lynx 4S recording system (Neuralynx) and data was analyzed off-line using custom written MATLAB scripts.

Acoustic stimuli (pure tones of varying frequency and intensity, 100 ms duration, 5 ms sine squared ramps, 150 ms inter-stimulus interval) were delivered with a near-field loudspeaker, as described for ABR recordings. Optical and electrical stimulation was achieved with the LED-based implants described in this manuscript and 4-channel, clinical-style electrical cochlear implants (MED-EL), respectively. Implants were inserted into the cochlea via a cochleostomy in the middle turn and driven by a custom-designed current source. Optical stimuli were delivered by individual LEDs and consisted of 1 ms light pulses of varying intensity. For electrical stimulation, biphasic (100 μ s phase duration) current pulses of varying intensity were presented. An external ball electrode in front of the bulla served as a reference for monopolar stimulation, while the neighboring stimulation electrode in basal direction served as a reference for bipolar electrical stimulation.

Raw data traces were filtered with a Butterworth filter (4th order, 0.6–6 kHz) and peaks exceeding a threshold (mean plus three median absolute deviations of the whole data trace) were registered as time stamps of neuronal events. After each time stamp, an artificial refractory time of 1 ms was implemented to not overestimate the responses. A linear interpolation from the sampling point just before trigger onset to the sampling point 3 ms after trigger onset was performed before thresholding in order to remove artefacts. The frequency tuning of multi-neuronal clusters was assessed by presenting pure tones (20–30 repetitions for each combination presented in pseudorandom order). The characteristic frequency was then defined as the frequency which elicited neural responses at the lowest sound pressure level during stimulation. For artificial auditory nerve stimulation (both optical and electrical), multi-unit spike rates were sorted into a response matrix according to electrode position and stimulus intensity. A discrimination index (d') was then calculated for each multi-unit (electrode) for increasing stimulus intensities, starting in the absence of stimulation (zero intensity condition). d' values were then summed up for successively increasing stimulus intensities in order to calculate a cumulative discrimination index. Based on the response matrix, iso-contour-lines were interpolated with MATLABs contour-function at integer d' values. As in previous publications (11, 33, 34), a d' value of 1 was considered as the threshold for neural activation, and the electrode with the lowest threshold was defined as the best electrode. The spread of excitation was then assessed as the distance between the dorsal- and ventral-most electrodes with responsive multi-units (i.e. a d' of 1 or higher) at the stimulus intensity where the best electrode reached a d' of 1.5, 2, 2.5 or 3. By quantifying neural responses in this activity-dependent manner, the strength of neural responses at the best electrode becomes the determinant of the intensity at which the spread of excitation is quantified (rather than choosing an absolute stimulus intensity), which enables a fair comparison across different stimulus modalities.

Statistical analysis

All data shown in the figures are expressed as mean $\pm$ SD, unless stated otherwise. Raw data for graphs containing an animal n lower than 20 are given in table S2. Unless noted otherwise, an α level of 0.05 was considered significant. Statistical analyses were performed using MATLAB R2016a and Python 3.8.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data and materials availability

All data associated with this study are present in the paper or the Supplementary Materials.

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One Sentence Summary

Optogenetic hearing restoration in rodents by LED-based multichannel optical cochlear implants provides improved spectral selectivity.

Fig. 1. Design of the LED-based multichannel optical cochlear implants.

(A) Reconstruction of the scala tympani of a rat based on X-ray tomography. Green line: center of scala tympani; blue areas: exemplary cross-sections, one of them with blue lines representing short and long axes; black line: modiolar axis of the cochlea subjectively derived from the X-ray data. Scale bar, 500 μm . (B) Radius of curvature (dotted line), short (light blue) and long (dark blue) axes of cross-sections (area in green, right axis) as a function of distance from the round window. (C) Design of the highly flexible PI-based linear array of ten LED chips integrated at the tip of the slender implantable

section of the oCI. The LEDs are individually wired and connected via a ZIF interface at the wider connector part comprising fixation holes for optional device attachment to the skull. **(D)** Design of the probe tip with resistive temperature sensor integrated beneath the first of four LED chips that were arranged at a pitch of 750 μm . The platinum meander of the temperature sensing element is connected via four wires for a precise resistance measurement. **(E, F)** Optical micrographs of fabricated and silicone-encapsulated oCIs with **(E)** ten LED chips assembled at an LED pitch of 350 μm and **(F)** with an LED pitch of 750 μm and with an integrated temperature sensor beneath the first LED. **(G)** Functionality test of an oCI demonstrating the individual control of LEDs prior to device passivation. **(H)** Bending test of a silicone-molded oCI inserted into a 300- μm -wide channel with an outer radius of 1 mm milled into a PMMA substrate.

Fig. 2. Electrical, optical, and thermal characterization of an oCI.

(A) V - I curves of ten LEDs of an oCI exhibiting a threshold voltage of 2.6 V. (B) Time-averaged radiant flux Φ of ten assembled and passivated LEDs of an oCI as a function of the LED current I_{LED} applied at 10 % DC and a frequency of 10 kHz. (C) Spectral density distribution of LED 1 with peak emission at 457 nm (black line) and ChR2-normalized action spectrum (blue line) (28) with a peak at 460 nm (29). (D) Modeled time-dependent temperature T of an oCI sample with integrated temperature sensor in response to current pulses of amplitude I_{LED} at 100% DC and duration $t_{\text{on}} = 4$ ms; the pulses produced the

indicated radiant flux values Φ ; the oCI was sandwiched between agarose gel layers kept at 37°C. **(E)** Modeled maximum temperature $T_{\max}$ vs. t_{on} , when the LED was driven with $I_{\text{LED}} = 38.9$ mA to $\Phi = 18.1$ mW. **(F)** $T_{\max}$ vs. Φ for $t_{\text{on}} = 1, 2,$ and 4 ms. **(G)** Simulated temperature profile of an oCI cross-section immersed in an aqueous medium (LED operated with $I_{\text{LED}} = 38.9$ mA and $t_{\text{on}} = 10$ ms). **(H)** Simulated transient temperatures (red circles: at position of Pt meander; green triangle: oCI top surface; blue stars: oCI side; bright blue diamonds: oCI bottom surface; purple triangle: 104 μm above the oCI) in comparison with the modeled temperature increase based on the Pt meander measurements (black squares). **(I)** Measured leakage currents I_{leak} vs. time of three representative oCI samples for various polymeric encapsulation procedures (Ar = argon plasma treatment for surface activation). See fig. S2A (up to stage vi). **(J)** oCI longevity derived from I_{leak} measurements, as shown in (I); oCI failure is defined by $I_{\text{leak}} > 1$ μA for more than ten consecutive measurement points. The temporal behavior of I_{leak} of samples marked by an asterisk are shown in panel G; dashed horizontal lines indicate a long-term measurement still running; vertical lines in each horizontal bar indicate the failure of a specific sample.

Fig. 3. oCI stimulation of the auditory pathway in Chr2 transgenic rats.

(A) Photograph of a silicone-encapsulated oCI connected to a PCB via a ZIF connector in comparison to a 200 µm optical fiber, magnified in the lower panel. (B) Retro-auricular approach to the middle ear: Surgical situs shows a bullostomy (dashed line) revealing the cochlea with the stapedial artery and round window. The inset shows the oCI inserted into the round window. (C) Three-dimensional model of a rat cochlea derived from X-ray tomography showing the position of the LED-based oCI in the scala tympani when inserted via the round window. The insertion depth was 5.1 mm, harboring 8 LEDs inside the scala tympani. Note that the cochlea was reflected horizontally relative to position in (B) for easier orientation. (D) Comparison of aABR and oABRs evoked by laser light (473 nm) and LED light (457 nm). Top: average of acoustic responses to 500 clicks (300 µs) at 60 dB (sound pressure level [SPL]) applied at 20 Hz. Middle: rectangular 4-ms-long laser stimulation at 6.8 mW and 10 Hz (average of 500 trials). Bottom: rectangular 4-ms-long LED stimulation at 6.6 mW and 10 Hz (average of 500 trials). First response peak (p1) is marked with an asterisk. (E) oABR recordings obtained from an LED stimulation (4 ms long duration at 8.9 mW and 10 Hz, 500 trials) with the LED placed inside the round window (dark blue), in the bulla outside the round window (light blue), the first LED at the tip of the array inside scala tympani after the animal was euthanized (red) and in a negative Chr2 rat (control, black): only onset and end artifacts are evident in both cases. (F) Representative oABRs obtained from five different LEDs (8-ms-long duration at 2.15 mW and 2 Hz, 300 trials) of an implanted oCI.

Fig. 4. Characterizing oCI stimulation by recordings of oABRs in rats.

Optical CIs or optical fibers were placed on the middle cochlear turn via a cochleostomy. (A) Exemplary oABRs driven by LED 1 of oCIs 1-5 (color coded) in the transgenic Chr2 rat used in (B to D) with a radiant flux of 18.1 mW (4-ms-long stimulus at 10 Hz, 500 trials throughout the figure). Note the onset and end artifacts of opposite polarity that flank the stimulus. First peak of response (p1) is marked with an asterisk. (B to D) oABR amplitude of p1 recorded from one rat with 5 different oCIs as a function of radiant flux (B, duration: 4 ms, rate: 10 Hz), stimulus duration (C, radiant flux 18.1 mW, rate: 10 Hz) and stimulation rate (D, 18.1 mW, duration: 2 ms). Negative standard deviation (SD) shown in light blue. (E) Exemplary oABRs from transgenic Chr2 rats 1-5 (color code) used in (F to H) stimulated by LED 1 of oCI 2. (F to H) p1 amplitude of oABRs recorded from four rats, first using a 200 μ m optical fiber (coupling in the light of a 473 nm laser; dark grey symbols), thereafter employing LED 1 of oCI 2 (blue symbols), presented as a function of radiant flux (F,

duration: 4 ms, rate: 10 Hz), stimulus duration (**G**, radiant flux: 18.1 mW (oCI), 27.3 mW (laser), rate: 10 Hz) and stimulation rate (**H**, radiant flux 18.1 mW (oCI), 27.3 mW (laser), duration: 2 ms). The oCI SDs are shown in light blue, laser SDs (only negative SDs shown) in light grey.

Fig. 5. Spectral selectivity of LED-based oCI stimulation of SGNs in gerbils.

(A to C) Peri-stimulus-time-histograms in response to maximum stimulus intensities of optogenetic (A), monopolar (B), and bipolar electrical stimulation (C). n = stimulation sites (LEDs or electrodes)/gerbils. (D to F) Exemplary spatial tuning curves of neural activation in the ICC in response to optogenetic (D), monopolar (E), and bipolar (F) electrical stimulation. (G) Tonotopic organization of the ICC: characteristic frequencies recorded at different electrodes as a function of recording depth. Tonotopic slopes were linearly fitted for each animal (indicated by different colors). N = multi-units/gerbils. p according to Pearson's correlation coefficient, calculated for all animals (H) Spectral

selectivity of optogenetic, mono- and bipolar electrical stimulation. Error bars indicate mean $\pm$ SEM. *** $P < 0.001$, repeated-measures ANOVA and post-hoc pairwise comparisons. Only statistically significant differences are indicated.

Fig. 6. Sound processor for driving optical cochlear implant (oCI).

(A) Flow chart of the battery-powered sound processor (highlighted in green) operation in the shuttle box paradigm experiments. The shuttle box control unit consisting of a PC with a digital-analogue/analyse-digital converter card and custom MATLAB software communicates over a radio protocol (highlighted in red) with a sound processor to deliver predefined optical stimulation to the oCI or generates an acoustic click stimulus (highlighted in yellow) captured by a sound processor triggering optical stimulation via the oCI. (B) Photograph of the rat with a base for a screwable processor housing (inner diameter: 20 mm) and a connector for mounting the sound processor. (C) Photograph of the oCI system showing a sound processor (circuit board diameter: 20 mm) and a battery mounted on the base fixed to the rat's head. The screwable cap was removed for illustration purposes.

Fig. 7. Chronic application of the complete multichannel LED-based oCI system in behavioral experiments in rats.

(A) Experimental workflow. AAV-CatCh was injected into the left cochlea at postnatal day 6. Several weeks later, acoustic ABRs were recorded and rats were trained in the shuttle box four times using acoustic stimulation. After oABR recordings, animals were deafened by bilateral intracochlear injections of kanamycin solution and implanted with an oCI. Subsequently, shuttle box experiments were performed using optical stimulation which was initiated by a pre-programmed sound processor. In subsequent sessions the processor was set

to transform acoustic stimuli into optical stimuli (in real time). Finally, rats were subjected to single shuttle box sessions with switched-off sound processor and aABR recordings confirming deafness of the animals. **(B)** Representative aABR in response to a 45 dB click stimulus before deafening (top) and representative oABR in response to laser fiber stimulation prior cochlea implant implantation. **(C)** Absence of aABRs in response to 110 dB for click stimuli (top) and 2, 8, and 32 kHz pure tones after deafening. Vertical dashed lines indicate stimulus onsets. All traces were recorded from the same animal. **(D)** Photo of rat inside the shuttle box (front wall removed) in a sound-attenuating chamber carrying the sound processor on its head. **(E)** Individual behavioral performance in shuttle box trials during initial acoustic and later predefined optical sessions. Lines show performance of the animal (correct response to target stimuli minus response to sham trials which represent baseline locomotion activity of the animal). Color of lines corresponds to individual rats. **(F)** Mean response rate [%] to hit (stimulus) and sham (no stimulus) trials in acoustic sessions when sound processor was turned on (left; n = 2, hit: 92 %, sham: 2.5 %) or off (right; n = 1, hit: 50 %, sham: 65 %) in one deaf, AAV-transduced, implanted animal.