Video Article

Optogenetic Stimulation of the Auditory Nerve

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Abstract

Direct electrical stimulation of spiral ganglion neurons (SGNs) by cochlear implants (CIs) enables open speech comprehension in the majority of implanted deaf subjects[1-6]. Nonetheless, sound coding with current CIs has poor frequency and intensity resolution due to broad current spread from each electrode contact activating a large number of SGNs along the tonotopic axis of the cochlea[7-9]. Optical stimulation is proposed as an alternative to electrical stimulation that promises spatially more confined activation of SGNs and, hence, higher frequency resolution of coding. In recent years, direct infrared illumination of the cochlea has been used to evoke responses in the auditory nerve[10]. Nevertheless it requires higher energies than electrical stimulation[10,11] and uncertainty remains as to the underlying mechanism[12]. Here we describe a method based on optogenetics to stimulate SGNs with low intensity blue light, using transgenic mice with neuronal expression of channelrhodopsin 2 (ChR2)[13] or virus-mediated expression of the ChR2-variant CatCh[14]. We used micro-light emitting diodes (μLEDs) and fiber-coupled lasers to stimulate ChR2-expressing SGNs through a small artificial opening (cochleostomy) or the round window. We assayed the responses by scalp recordings of light-evoked potentials (optogenetic auditory brainstem response: oABR) or by microelectrode recordings from the auditory pathway and compared them with acoustic and electrical stimulation.

Video Link

The video component of this article can be found at https://www.jove.com/video/52069/

Introduction

According to the World Health Organization, 360 million people worldwide suffer from hearing loss. In deaf subjects, direct electrical stimulation of SGNs by CIs enable open speech comprehension in the majority of them[1,2,4,5]. Even though CIs have been implanted in more than 200,000 people, therefore being the most successful neuroprosthesis, sound encoding driven by the current cochlear implants is limited. CIs are based on electrical stimulation by a certain number of electrodes where each one activates a tonotopic region of the auditory nerve thus bypassing the dysfunctional sensory organ of Corti in the cochlea. Normal hearing listeners can discriminate more than 2,000 frequencies, however today's CIs use only up to 12-22 frequency channels[4]. This is due to widespread current flow from each stimulating electrode[7,9], activating a large number of SGNs that represent many different sound frequencies[8,15]. This limitation can be improved using multipolar stimulation but at the expense of higher power consumption[16,17]. Their output dynamic range for sound intensity is also limited, typically below 6-20 dB[4,18]. For these reasons, improving frequency and intensity resolution are important objectives for increasing CI performance to ameliorate speech recognition in noisy environments, prosody comprehension and music perception.

A different option to stimulate the auditory nerve is optical stimulation. Light can be conveniently focused to target a small SGN population, promising better spatial confinement, increasing frequency resolution and also widening dynamic range, resulting in a better intensity resolution. Indeed, cochlear stimulation with infrared light has shown excellent frequency resolution in animal models[10,11,19]. One disadvantage of this kind of stimulation is that it requires higher energies than electrical stimulation[10,11]. Moreover, concerns about the ability of the method to directly stimulate auditory neurons have been raised[12,20].

As an alternative to infrared stimulation, we employ optogenetics to render SGNs light sensitive. Optogenetics is a novel approach that combines genetic and optical techniques to non-invasively and specifically control cells with high temporal precision (reviews[21-23]). The currently most frequently used modality employs the expression of the microbial channelrhodopsin 2 (ChR2) gene of *Chlamydomonas reinhardtii* and variants thereof, encoding a light-gated cation channel[24]. ChR2 is a 7-transmembrane-helix protein that, when transduced into neurons and activated by blue light, acts as non-selective cation channel, thus depolarizing the cells[24-27]. ChR2 has been well characterized[24,28-31] and many variants have been developed to modify action spectrum, gating and permeability properties[32,33]. The aim of our work is to establish cochlear optogenetics for the activation of the auditory pathway. We note that the optogenetic approach to stimulate the auditory nerve requires genetic manipulation of the spiral ganglion for the expression of channelrhodopsin. Working with mice and rats allows the use of available transgenic animals[13,34,35], which

provide expression of channelrhodopsin with little variability along the tonotopic axis and across animals[36]. Combining conditional alleles[37] with appropriate Cre-lines provides for cell-specific expression. Gene transfer into the spiral ganglion of other animals requires the use of virus such as adeno-associated virus that is a standard approach in optogenetics[38] and that we showed to work well in mice[36]. Genetic manipulation and expression of transgenes encoding alien proteins bear risks for adverse effects such as immune responses and/or proliferation, compromised condition or even death of genetically manipulated cells. For the purpose of this demonstration we use transgenic mice expressing ChR2 in spiral ganglion neurons under the Thy-1 promotor[13] to optically stimulate the auditory pathway. We note that other channelrhodopsin variants can be used for the same purpose as we demonstrated using virus-mediated transfer of the variant CatCh[14] into SGNs[39].

While cochlear optogenetics requires genetic manipulation, it offers molecular tuning for optimized SGN stimulation and promises improved frequency and intensity resolution when compared to electrical stimulation. Optogenetic stimulation of the auditory pathway is highly relevant for hearing research. For example, it promises advances in studies of the activity-dependent refinement of tonotopy during development, in the analysis of the requirement for spectral integration in sound localization and of the extent of interaction between frequency-specific afferent projections in the central auditory system.

Protocol

All experiments presented in this work were conducted with the ethical standards defined by the German law for the protection of experimental animals. The University of Goettingen board for animal welfare and the animal welfare office of the state of Lower Saxony approved the experiments.

1. Preparation of µLED-stimulator

1. For µLEDs, first prepare the µLED-stimulator. Use blue LEDs with 200 by 200 µm active surface (µLED, see Materials Table).
2. Solder wires to the µLED. Then, encapsulate the µLED and the connections using epoxy glue. Leave the device overnight to let the epoxy cure.
3. For mechanical stability and electrical insulation funnel the wires through a thin glass capillary, use 1 mm outer diameter borosilicate capillaries and seal the junction with epoxy (**Figure 1D**) such that the capillary can be mounted on a mechanical manipulator.
   NOTE: There is a trade-off of making the capsule thick enough to get a good electrical isolation (avoiding stimulation artifacts), but thin enough to let the LED move freely inside the bulla and to locate it onto the cochleostomy. It is a good practice to prepare more than one LED.
4. To confirm good electrical isolation, first prepare a Petri dish with 0.9% NaCl and dip 3 bare ended copper wires into the liquid. This serves to simulate an animal with ABR electrodes. Connect the wires to the ABR-amplifier and from there to an oscilloscope.
5. Then, connect the LED to the stimulator and immerse it into the solution until it is totally covered. Drive the LED with pulses (see step 3.1.1) at the maximum allowed current for longer than 20 min and check for stimulation artifacts. If they appear, discard the LED and try a new one.

2. Surgical Procedure

1. Use 4-10 week-old transgenic mice expressing ChR2 under the Thy1.2 promoter (line 9 in Wang *et al.*[13]). To manipulate the animal and perform the surgery, always use clean and well identified surgical tools.
2. Anesthetize animals with an i.p. injection of a mixture of urethane (1.32 mg/kg), xylazine (5 mg/kg), and buprenorphine 0.1 µg/g diluted in 0.9% sterile NaCl solution and place the mouse onto a heating plate for maintaining body temperature at 37 °C. With this dose, anesthesia typically lasts for the whole experimental protocol.
   1. Beginning 10 min after initial anesthesia induction check for signs of arousal by the paw withdrawal reflex. In case of movements, apply maintenance doses of urethane (.66 mg/kg) and buprenorphine (0.05 µg/g) diluted in 0.9% sterile NaCl solution (without Xylazine). Again check the paw withdrawal reflex and proceed only if the reflex is absent.
3. Carefully shave the retroauricular area in preparation of the incision. Use a retroauricular approach to reach the bulla (air-containing middle ear cavity).
   1. With a micro forceps carefully dissect and/or remove part of the neck muscles, *e.g.,* platysma, sternocleidomastoid, and scalenes.
   2. Find and expose the bulla (**Figure 1A**). Identify the bulla as a bony structure with a characteristic spherical shape with a ring on the bulla surface that indicates the insertion of the tympanic membrane (**Figure 1A**). Make a hole with the tip of an insulin needle just below this ring. Start there, remove the bone that covers the bulla with a fine Rongeur (gnawer) or microforceps in such a way that the promontorium of the cochlea is exposed (**Figure 1B**).
4. For the cochleostomy, gently shave off the bony capsule to open a small window (cochleostomy: ~500-800 µm), taking care to leave intact the membranous labyrinth (**Figure 1B**). Perform the cochleostomy on the second cochlear turn as it is far enough from the stapedial artery and from the apex. Opening the apex could produce rupture of the cochlea including a fracture of the modiolus.
5. For a round window insertion of an optical fiber or µLED-implant to scala tympani, carefully enlarge the round window niche by scratching the bony niche with the tip of a sharp scalpel (a blade number 11 is a good option). Always use a new blade or scalpel. Be very careful as the stapedial artery runs very close to the round window (**Figure 1B**).

3. Optical Stimulation Using Two Approaches

1. For transcochlear stimulation, use µLEDs or a fiber coupled laser.
   1. Mount the capillary carrying the µLED on a mechanical manipulator and carefully position the µLED onto the cochleostomy. Use oABR, elicited by 3-10 msec long stimulation from a current source (typically 5-40 mA at 2.7 V) at 1-5 Hz, as a means to optimize the position and orientation of the µLED.
   2. For laser stimulation, use a 473 nm continuous wave laser and a 250 µm optical fiber (see Materials). Couple the optical fiber to a blue laser source that ideally offers fast analogue control of power (else use an acousto-optical device or a fast shutter to define the

optical stimulus). Using a micromanipulator, locate the tip of the fiber directly onto the cochleostomy and fix it there using dental cement (transcochlear stimulation).

2. For intracochlear stimulation, insert the fiber through the round window into the scala tympani. Then use 3-10 msec laser pulses of 10-30 mW (power of fiber output) at 1-5 Hz to elicit oABR. The large amplitude of oABR enables the experimenter to observe oABR evoked by single stimuli on the oscilloscope for optimizing the position and orientation of the emitter (µLED or fiber) using oABR amplitude as a read-out.
3. Once good oABR have been achieved, fix the fiber with cyanoacrylate glue or dental cement and wait until the glue is solid.
NOTE: It is normal to observe some liquid coming out from the cochlea. However to avoid problems with the polymerization of the cement, it is advisable to dry up the region using fine cotton wicks.

4. Recordings of oABR

1. Insert needle electrodes underneath the pinna, on the vertex and on the back near the legs and connect them to the amplifier.
2. Amplify the difference potential between vertex and mastoid subdermal needles. Sample at a rate of 50 kHz and filter (1-10,000 Hz). Visualize the difference potential on an oscilloscope ideally near the recording setup for optimizing the position of the optical stimulator. Use signal averaging (50-1,000x) and store the data on a computer for offline analysis.

5. Recordings of Optically Evoked Local Field Potentials

1. Place the animal into a stereotactic frame. Using forceps centrally pull the skin overlying the cranium and perform a median incision with scissors roughly extending from between the eyes to the point where the neck muscles attach to the skull.
    1. Reflect the skin laterally. Cut the periosteum lengthwise along the sagittal suture and remove it sideward by gently rubbing the exposed cranial bone with a cotton swab.

2. Position a custom made metal plate onto the exposed skull leaving approximately 1 mm space between the caudal end of the plate and lambda. With a 0.7 mm diameter drill carefully drill a hole into the parietal bone.
3. Gently screw in a 0.85 mm diameter screw – to serve as reference for potentials recorded from the inferior colliculus (IC) – holding the screw with forceps as long as the screw is not securely fastened. Ensure good contact between screw and dura without penetrating the brain. Glue the screw and the metal plate onto the skull with cyanoacrylate glue.
4. Loosen the neck muscles gently from the skull. Pull the neck muscles caudally for circa 1-2 mm. Identify the intersection of the superior sagittal sinus (SSS) and the transverse sinus (TS). If necessary, increase the transparency of the skull by moistening the bone with physiological NaCl solution.
    1. Identify the IC in mice as lying directly caudal to the SSS and TS intersection. After identification of the location of the IC slowly and carefully perform a trepanation extending ca. 0.5 mm rostrally to 1.5 mm caudally to the TS and roughly 3 mm laterally.
    2. Avoid any injury to the SSS and TS as these would preclude a successful experiment. Here, test whether the trepanned bone has become loose by softly pushing it. Using forceps slowly lift off the bone.

5. With an appropriate adaptor attach a multichannel array and headstage onto a micromanipulator. To prevent destroying the multichannel recording array during insertion into the IC, carefully remove the dura with a bent tip of a small hypodermic needle starting close to a bone edge.
    1. Remove the animal from the stereotaxic frame. Fix the metal plate onto a bar to remain stereotaxic fixation. Position the recording probe over the IC and insert it under microscopic control such that the top most channel is just visible at the surface.

6. Amplify the difference potential between the individual channels of the recording array and the reference screw in the parietal bone, sample at a rate of 32 kHz, low-pass filter (300 Hz) and store on a computer for offline analysis.
7. All animals should be humanely euthanized at the end of the procedure prior to anesthetic recovery in accordance with relevant euthanasia guidelines. Suitable methods of euthanasia may include cervical dislocation or CO_2 inhalation.

Representative Results

An optimal cochleostomy is critical and increases the probability of a successful experiment**.** This means the window is regular, small, and there is no injury of the internal cochlear structures. For example, bleeding indicates damage of the stria vascularis. A good example is presented in **Figure 1B**.

Using ChR2-transgenic mice, ChR2 is expressed in the SGNs within the cochlea (**Figure 1C**). Blue light illumination, either by µLED or laser, elicits large oABR, which differ from acoustic ABR (aABR) in amplitude and waveform. oABR (**Figure 1F**) have larger amplitudes than aABR (**Figure 1E**) but they are comparable to electrical ABR (eABR, **Figure 1G**)**.** This can be interpreted as oABR and eABR reflecting recruitment of more SGNs and/or a more synchronized activation of SGNs than for sound-evoked aABR.

To elicit eABR a rodent electrical CI (MED-EL) was inserted into the round window. We used biphasic current pulses (80 µsec phase duration, 20 µsec interphase duration, 500 µA, 6 Hz stimulation rate) to stimulate and averaged responses to 100 stimulus repetitions. Stimulation with 2 glass-insulated tungsten electrodes (1 inside and 1 outside of the cochlea) is also possible.

It is important to note that oABR are not detectable in wild type animals, after inhibition of voltage-gated sodium channels or after sacrificing the animals[39]. These control experiments render heating as a mechanism underlying optically evoked ABRs unlikely.

We verified propagation of the activity through the auditory pathway by extracellular recordings in the central auditory system. For an example, optogenetic stimulation of the cochlea elicited also neural activity in the auditory midbrain (inferior colliculus, **Figure 2**).

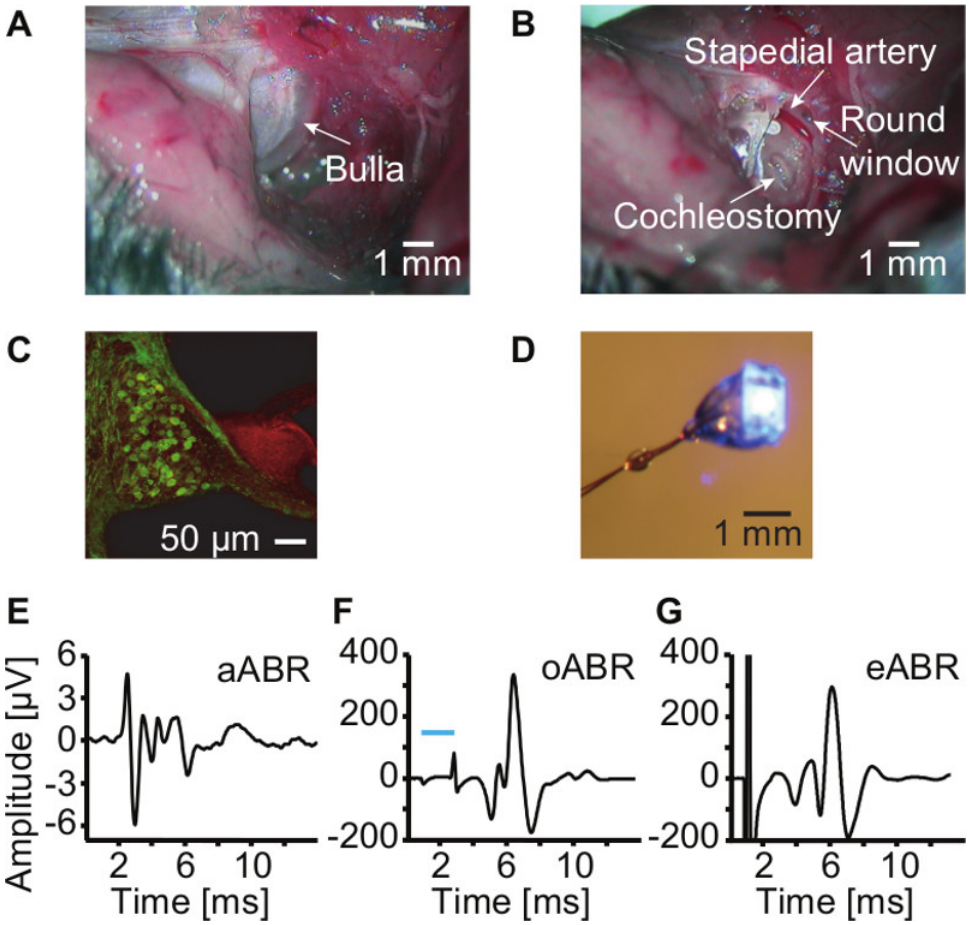

Figure 1: Surgical procedure, µLED preparation, and oABR recording. (**A**) Dissecting the neck muscles exposes the bulla; observe its characteristic spherical shape and the ring where the eardrum inserts into the bone, which can be used as a landmark. Scale bar 1 mm. (**B**) Opening of the bulla will expose the promontorium. The cochleostomy was performed on the second cochlear turn. Other landmarks are indicated, among them the stapedial artery. Scale bar 1 mm. (**C**) ChR2-YFP expression in SGNs. Immunolabeling for GFP (green) and phalloidin-AF-568 (red). Scale bar 50 µm. (**D**) µLED, wired but not yet funneled into the capillary. Scale bar 1 mm. (**E**), (**F**), and (**G**) representative examples of aABR (click, 20 Hz, 80 dB), oABR (2 msec, 1 Hz, 4 mW/mm^2) induced by LED stimulation and eABR (900 µA, 20 Hz) via glass-insulated tungsten electrodes. Please click here to view a larger version of this figure.

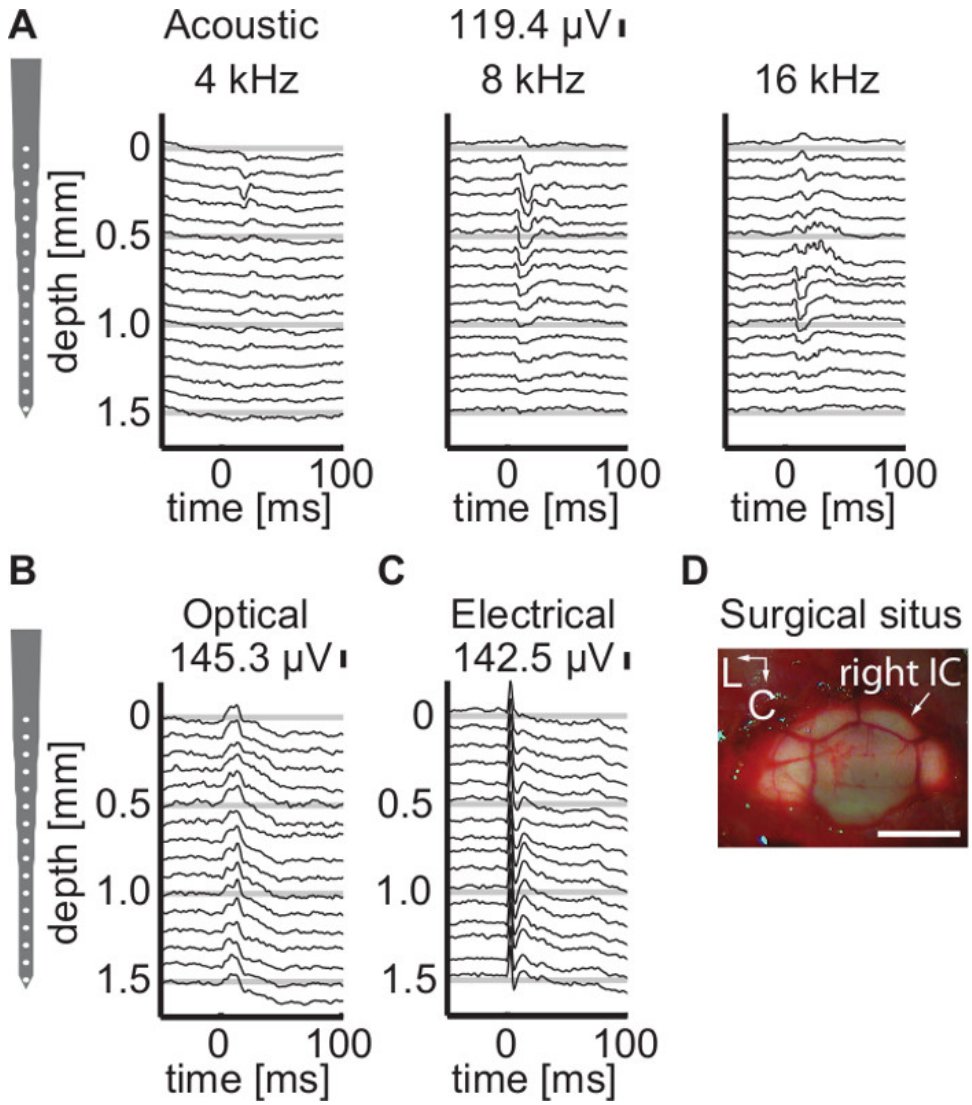

Figure 2: Single trial local field potentials recorded from the IC after acoustic, optogenetic, and electrical cochlear stimulation. A schematic representation of the multichannel recording probe is drawn on the left side of each panel. (**A**) Single presentations of pure tones with various frequencies (80 dB SPL) lead to a distinguishable gradient of activity with lower frequencies leading to more superficial activation than higher frequencies. This observation is used to evaluate successful insertion of the multichannel recording array into the IC. (**B**) Optical stimulation (24 mW, 6 msec duration) and (**C**) Electrical stimulation (biphasic, 80 µsec phase duration, 20 µsec interphase duration, 500 µA) also led to local field potentials in the IC often exhibiting comparable response amplitudes. (**D**) Surgical situs including both IC as well as a portion of the cerebellum. Scale bar 5 mm. Please click here to view a larger version of this figure.

Discussion

The described experiments demonstrate the optogenetic stimulation of the SGNs, and can, in principle, also be used to stimulate inner and/or outer hair cells, provided the expression of opsins. These experiments require much patience and care. As mentioned before, the most critical steps are a good cochleostomy/round window insertion as well as an appropriate position and orientation of the light source.

There are limitations with optogenetic stimulation when using ChR2. In our case oABR amplitude increases with light intensity and pulse duration but decreases when rate of stimulation increases[15]. Response latency also depends upon light intensity, decreasing when the intensity of the stimulus increases. We propose that these responses are the result of the kinetics of the ChR2 and the number of channels expressed in the SGNs. In addition, ChR2 can cause extra spikes, failures and membrane potential summation at high spike rates[27,40]. There have been continuous efforts to overcome these limitations[32,33]. We have recently reported the use of virus-mediated expression of the ChR2-variant CatCh[14] in SGNs, which reduced the light requirement and allowed spiking to higher rates of stimulation[39]. Most recently the Boyden laboratory reported on Chronos, a channelrhodopsin with faster kinetics and high light sensitivity[41].

We propose that optogenetic stimulation of the auditory pathway can contribute to auditory research and, in the future, to cochlear prosthetics. We consider that it can improve speech recognition and perception of prosody and music. Translation of this approach into the clinic requires

many critical developments such as the optimization of channelrhodopsins, the development of multichannel optical stimulation technology for an optical cochlear implant as well as the demonstration of biosafety for optical stimulation and long-term genetic manipulation of SGNs.

Disclosures

The authors declare that they have no competing financial interests.

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