Attenuated infrared neuron stimulation response in cochlea of deaf animals may associate with the degeneration of spiral ganglion neurons

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Abstract: Hypothesis: We hypothesize that degenerated spiral ganglion neurons (SGNs) in guinea pigs reduces auditory brainstem responses evoked by pulsed infrared stimulation. Background: Pulsed infrared laser excitation can directly evoke physiological responses in neuronal and other excitable cells in vivo and in vitro. Laser pulses could benefit patients with cochlear implants to stimulate the auditory system. Methods: Pulsed infrared lasers were used to study evoked optical auditory brainstem responses (oABRs) in normal hearing and deafened animals. Also, the morphology and anatomy of SGNs in normal hearing and deafened guinea pigs were compared. Results: By recording oABRs evoked by varying infrared laser pulse durations, it is suggested that degeneration of SGNs in deafened guinea pigs was associated with an elevated oABR threshold and with lower amplitudes. Moreover, oABR threshold decreased while amplitudes increased in both normal hearing and deafened animals as the pulse duration prolonged. Electron microscopy revealed that SGNs in deafened guinea pigs had swollen and vacuolar mitochondria, as well as demyelinated soma and axons. Conclusion: Infrared laser pulses can stimulate SGNs to evoke oABRs in guinea pigs. Deafened guinea pigs have elevated thresholds and smaller amplitude responses, likely a result of degenerated SGNs. Short pulse durations are more suitable to evoke responses in both normal hearing and deafened animals.

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References and links

1. V. Goyal, S. Rajguru, A. I. Matic, S. R. Stock, and C. P. Richter, “Acute damage threshold for infrared neural stimulation of the cochlea: functional and histological evaluation,” Anat. Rec. (Hoboken) 295(11), 1987–1999 (2012).
2. C. P. Richter, A. I. Matic, J. D. Wells, E. D. Jansen, and J. T. Walsh, Jr., “Neural stimulation with optical radiation,” Laser Photon. Rev. 5(1), 68–80 (2011).
3. A. D. Izzo, C. P. Richter, E. D. Jansen, and J. T. Walsh, Jr., “Laser stimulation of the auditory nerve,” Lasers Surg. Med. 38(8), 745–753 (2006).
4. M. G. Shapiro, K. Homma, S. Villarreal, C. P. Richter, and F. Bezanilla, “Infrared light excites cells by changing their electrical capacitance,” Nat. Commun. 3(736), 736 (2012).
5. J. Wells, C. Kao, K. Mariappan, J. Albea, E. D. Jansen, P. Konrad, and A. Mahadevan-Jansen, “Optical stimulation of neural tissue in vivo,” Opt. Lett. 30(5), 504–506 (2005).
6. S. M. Rajguru, C. P. Richter, A. I. Matic, G. R. Holstein, S. M. Highstein, G. M. Dittami, and R. D. Rabbitt, “Infrared photostimulation of the crista ampullaris,” J. Physiol. 589(6), 1283–1294 (2011).
7. M. W. Jenkins, A. R. Duke, S. Gu, H. J. Chiel, H. Fujioka, M. Watanabe, E. D. Jansen, and A. M. Rollins, “Optical pacing of the embryonic heart,” Nat. Photonics 4(9), 623–626 (2010).
8. A. D. Izzo, J. T. Walsh, Jr., H. Ralph, J. Webb, M. Bendett, J. Wells, and C. P. Richter, “Laser stimulation of auditory neurons: effect of shorter pulse duration and penetration depth,” Biophys. J. 94(8), 3159–3166 (2008).
Introduction

Optical technologies play an increasingly important role in neuroscience as well as in developing treatments for neurological, psychiatric, and cardiovascular diseases. Importantly, optical technologies provide many advantageous features when compared to electrical stimulation, namely greater spatial resolution, accuracy, and no stimulation artifacts [1–3]. Pulsed infrared lasers have been applied directly to evoke physiological responses in neuronal and other excitable cells both in vivo and in vitro without any prior genetic or chemical manipulation [4, 5]. The targets of infrared lasers include peripheral and cranial motor nerves, vestibular hair cells, cochlear nerves, and the heart [6–10]. The advantage of direct infrared excitation makes it attractive for a variety of applications in basic and clinical research, ranging from hearing restoration to optical pacing [2].

Contemporary cochlear implants (CIs) are considered the most successful neural prostheses; they apply electrical currents that bypass damaged cochlear hair cells to stimulate spiral ganglion neurons (SGNs) directly, restoring the hearing in severely-to-profoundly deaf individuals, resulting in language and speech improvement [11]. While current CIs work well in quiet environments, patients with CIs find conversation in noisy environments difficult [12, 13]. One reason is that the electrical current spreads widely along the scala tympani and modiolus, and especially in noisy environments, interactions and broadly overlapping stimulations between electrodes can limit speech recognition and frequency resolution [14–16]. As an alternative to electrical stimulation, patients with CIs could be benefit from infrared laser stimulation applied in the auditory system. Previous studies demonstrated that laser-evoked optical auditory brainstem responses (oABRs) and compound action potentials (CAPs) could be recorded in both normal hearing and deafened animals [15]. Pulsed infrared radiation also activates sensory hair cells of the semicircular canal crista ampullaris of the toadfish in vivo [6]. Modulation of various parameters of infrared laser stimulation (wavelength, pulse duration, laser power and frequency) have demonstrated that infrared pulses are effective stimuli to evoke CAPs and oABRs through delivery of an optical fiber into the cochlea [1, 14, 17]. But the mechanism of optical stimulation-evoked CAPs and oABRs is still unclear. To confirm if either cochlear hair cells or SGNs are the targets of pulsed infrared stimulation resulting in CAPs and oABRs, further investigation is required. The safety and availability of infrared laser stimulation is important for developing CIs. In current study, we explored the energy required to evoke oABRs in both normal hearing and deafened guinea pigs, and determined the correlation between acute hearing loss and the oABR. We also investigate the ultrastructural changes of SGNs in deafened guinea pigs and how they might influence oABRs, and then determine the correlation between acute hearing loss and the oABR.

Materials and methods

Animals

Adult guinea pigs (both male and female) were used. Animal weights ranged from 250 to 300 g. All animals were assessed by acoustically evoked auditory brainstem responses (aABRs) at 8, 16, 24, and 32 kHz. Then oABRs were recorded in both normal hearing and deafened guinea pigs.

The care and use of the animals in this study were carried out in accordance with the National Health and Family Planning commission of the People’s Republic of China Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of Eye & ENT Hospital, Fudan University.

Deafening procedure

Guinea pigs were systemically deafened using a combination of kanamycin sulfate and furosemide. Kanamycin (400 mg/kg) was injected subcutaneously followed by slow
intravenous infusion of furosemide (100 mg/kg) as a loop diuretic. aABRs were measured prior to deafening procedures and at one-week post-deafening. Hearing loss is defined as a threshold shift of 40dB SPL between the two measurements [18]. Severe sensorineural hearing loss results in no aABRs at 93dB SPL in both ears [19].

**Acoustically evoked auditory brainstem response**

After the animals were fully anesthetized by an intraperitoneal injection of ketamine (60 mg/kg) and xylazine (4 mg/kg), aABRs were measured using TDT3 systems (modules RP6, PA5, Tucker-Davis Technologies, Inc. USA). Body temperature was maintained at 38°C by placing the animals on a heating pad (Inst. of Biomedical Engineering, China). Stainless steel electrode needles were used to record aABR, the positive electrode was inserted into subcutaneous of the vertex of the head, and the negative electrode was inserted into subcutaneous of inferior posterior of the ipsilateral auricle, while the grounding electrode was inserted into the apex of nasal. The acoustic stimuli were 10 ms tone bursts (including a 1-ms rise time and 1-ms fall time), at 8, 16, 24, and 32 kHz. The overall noise during recording was reduced by band-pass filtering, with the high-pass filter cutoff frequency set to 300 Hz and the low-pass filter cutoff frequency set to 3000 Hz; the amplifier gain was set to 20 dB.

**Animal surgery and preparation for stimulation**

Guinea pigs were anesthetized as above, and they were maintained with one third of the primary doses throughout the procedures whenever animals showed signs of increasing arousal, which was assessed every 30 min by a paw withdrawal reflex. After the animals were fully anesthetized, their body temperature was maintained at 38°C by placing the animals, ventricumbent position, right ear up, on a heating pad (Inst. of Biomedical Engineering, China). Animal heads were stabilized on a head stereotaxic apparatus (SR-6N Stereotaxic Instrument, Narishige Scientific Instrument Lab, Japan). A C-shaped retroauricular skin incision was made behind the right ear, and the muscles attached to the bulla and to the styloid bone were carefully dissected and removed to expose the right bulla. Then, the bulla was opened to access the scala tympani of the cochlea.

**Pulse infrared laser stimulation**

Cochleostomy of the basal turn of the cochlea was done with a 0.5-mm-diameter diamond bur (H6Z130, Surgical Instruments Factory, China) approximately 0.5 mm anterior and inferior to the bony rim of the round window. After the exposure of the scala tympani of the basal turn, a flat polished 200-μm diameter optical fiber (NA = 0.22) was inserted through the cochleostomy into the scala tympani (Fig. 1(a)) and allowed recording of oABRs in normal hearing and deafened guinea pigs. The optical fiber was coupled to a benchtop diode infrared laser (SFOLT Co., Ltd., Shanghai, China). The distal end of the fiber was positioned at a distance of ~500–800 mm over the modiolus of the cochlea to stimulate SGNs. The output wavelength of infrared laser diode was 1850 nm, pulse durations of 150, 100, 90, 80, 70, 60, and 50 μs were applied at a pulse frequency of 10 Hz. The pulse energy of the laser was controlled directly by varying the current to the diode. The energy per pulse was measured in air at the distal end of the optical fiber with a J50LP-1A energy sensor (Coherent, Santa Clara, CA). Power ranged between 0 and 350 mW (0-35 μJ/pulse at 100 μs pulse duration).

The oABRs were also recorded by Tucker Davis Technologies TDT3 systems; the acoustic stimulus signals (10 ms) were captured from the output port of the PR6 module, and converted into a synchronous current pulse to trigger the diode laser generating an infrared laser pulse, which is synchronous with acoustic stimulus signals to stimulate SGNs in the basal turn. The acquisition parameters were the same as the parameters during aABR recording.
Fig. 1. a: Image of surgery approach (right ear), showing the round window, rim of the round window (pink star) posterior wall of the external auditory canal (pink triangle) and optical fiber; b: Schematic diagram of the optical fiber inserted into the scala tympani. c, d: Waveforms of oABR records when the laser located on different site (as the schematic diagram showing as b). Only when the laser located on spiral ganglion region, oABR could be recorded (showing as c).

**Preparation of specimens**

At the end of the electrophysiological experiments, the guinea pigs were deeply anesthetized and decapitated. The bullae were removed and the cochleae exposed. A small hole was made in the apex, and for immunofluorescence staining, cochleae were perfused and fixed with 0.1 M phosphate buffer solution containing 4% paraformaldehyde. For transmission electron microscopy (TEM), the cochleae were perfused and fixed with 2.5% glutaraldehyde. After a 12h fixation, the specimens were transferred into 0.01 M phosphate buffer, rinsed 3 times for 15 min then placed in 0.2 M EDTA for decalcification in 4°C, and EDTA was replaced every day for about 2 weeks until the cochleae could be easily cut.

**Immunofluorescence**

In order to compare the differences of hair cells and SGNs between pre- and post-deafened, Tuj1 and Myosin VIIa was used to stain the SGNs and hair cells respectively. After decalcification, specimens were embedded in optimal cutting temperature compound (O.T.C.) prior to making frozen sections. Specimens were permeabilized prior to all immunostaining with 0.5% Triton X-100 in PBS for 1 h at 37°C. All specimens were pre-treated for 30 min at room temperature with 10% goat serum in PBS blocking solution before the primary antibody was added. Specimens were then incubated with different primary antibodies diluted in 5% bovine serum albumin (BSA) in PBS overnight at 4°C. The following primary antibodies and dilutions were used: β-tubulin III (Tuj1, rabbit, 1:500, MMS-435P, Covance) and myosin...
VIIa (mouse, 1:200, 25-6790, Proteus Biosciences). Thereafter, appropriate fluorescent secondary antibodies, goat anti-rabbit Alexa Fluor 488 (1:400) and goat anti-mouse Alexa Fluor 555 (1:400), were applied overnight at 4°C followed by incubation with 4’,6-diamidino-2-phenylindole (DAPI, 1:800) for 10 min. All images were collected using a Leica TCS SP5 confocal microscope (Germany).

Transmission electron microscopy
Modiolus specimens with SGNs were fixed with osmium tetroxide for 2–3 h after decalcification, and then dehydrated with an ethanol gradient and embedded in pure epoxy propane. Specimens were cut into 70-nm slices with an ultramicrotome prior to staining by 3% acetic acid uranium/lead citrate. Images of the SGNs were captured by TEM (JEOL-1230, 80KV, Japan).

Statistical analysis
Means and standard deviations were calculated for the energy thresholds and amplitudes of oABRs. An analysis of variance (ANOVA) was performed to compare data within groups of both normal hearing and deafened animals. Kruskal-Wallis H tests were used when the data were non-normally distributed. If the Kruskal-Wallis H test indicated differences among the means, a posteriori test, Tamhane’s T2, was used for making pairwise comparisons among the means. Results were considered statistically significant if \( p < 0.05 \). All statistical tests were performed with IBM SPSS 18.0 statistics software.

Results
Deafening increases aABR threshold
A total of 24 adult guinea pigs were assessed for aABR with normal hearing prior to any intervention (Fig. 2). The thresholds of aABR were between 21.3 and 33.6 dB SPL on average in both ears at all frequencies tested (Table 1). Twelve guinea pigs were systemically deafened using a combination of kanamycin sulfate and furosemide. One of these 12 animals died two days after the treatment therefore excluded from the study. The aABR thresholds of the remaining 11 guinea pigs were dramatically elevated one-week post-treatment (Fig. 2). The average threshold for each frequency was higher than 93 dB SPL in both ears, except at 8 kHz, which had an average threshold of 83.3 dB SPL in the left ear and 84.6 dB SPL in the right ear (Table 1).
Fig. 2. The thresholds of aABR in normal hearing and deafened animals. Deafening (gray and red curves) significantly elevated hearing thresholds compared to normal hearing controls (brown and violet curves).

Table 1. The hearing threshold of hearing (control) and deafened animals (aABR/dB SPL)

| Side                  | 8 kHz         | 16 kHz        | 24 kHz        | 32 kHz       |
|-----------------------|---------------|---------------|---------------|--------------|
| Left Ear (Normal)     | 22.7 ± 2.9    | 22.9 ± 3.3    | 24.0 ± 2.9    | 33.8 ± 3.7   |
| Right Ear (Normal)    | 21.3 ± 2.2    | 23.1 ± 3.6    | 25.8 ± 4.1    | 32.7 ± 3.6   |
| Left Ear (Deafened)   | 83.3 ± 11.4   | 94.6 ± 4.7    | 97.9 ± 2.6    | 100 ± 0      |
| Right Ear (Deafened)  | 84.6 ± 11.4   | 96.7 ± 3.8    | 97.5 ± 2.6    | 100 ± 0      |

Table 1. The hearing threshold (aABR) of normal hearing and deafened animals.

Deafening increases oABR threshold while reducing amplitudes

OABRs were evoked and recorded with infrared laser pulses at different pulse durations in all 12 normal hearing and 11 deafened guinea pigs. Only when the optical fiber pointed to the Rosenthal’s canal, which means that the laser targeted to the spiral ganglion neurons region, can the oABRs be recorded in both normal hearing and deafened animals, no detectable oABRs could be recorded, neither the optical fiber pointed to the basilar membrane nor any other directions (Fig. 1(b), 1(c), 1(d)). The wave patterns of oABRs were similar between normal hearing and deafened animals; however, the waves appeared with lower noise signal and had larger amplitudes in hearing guinea pigs than deafened guinea pigs under the same stimulus parameters (Fig. 3). Moreover, the wave patterns of the aABRs and oABRs in normal hearing and deafened guinea pigs were similar.
Fig. 3. The wave patterns of aABRs and oABRs, all these results were recorded from the right ear of guinea pig, a is the trace of an aABR from a normal hearing guinea pig, b is the wave forms of an oABR from a normal hearing animal, and c is from the deafened guinea pig stimulated with a 100-μs pulse at 10 Hz at 0–35 μJ (the energy detected at the distal of optical fiber in the room temperature air was 0-31.5 μJ / pulse). The amplitudes were smaller in deafened animals (smaller amplitude scale, left axis), and the energy threshold of deafened animals was higher than normal hearing animals.
Next, we used optical radiation pulses to determine the thresholds of oABRs, that is, the lowest energy required to evoke oABRs by stimulating SGNs. The energy thresholds of oABRs in normal hearing guinea pigs increased as the pulse duration increased, especially when the pulse duration was longer than 100 μs. The average energy thresholds of oABRs increased significantly from 6.41 ± 1.35 μJ to 17.19 ± 3.69 μJ as the pulse duration increased from 50 μs to 150 μs. Although we observed a similar trend for deafened guinea pigs, the thresholds increased for all pulse durations (Fig. 4(a), Table 2). However, in both normal hearing and deafened animals, there were no significant differences between the thresholds when the pulse duration increased from 50 μs to 100 μs (normal hearing animals, \( p = 0.067 \); deafened animals, \( p = 0.164 \)).

The amplitudes of oABRs in response to various pulse durations in both normal hearing and deafened guinea pigs are shown in Fig. 4(b) and Table 3. Under same stimulus conditions, the amplitudes of oABRs in deafened guinea pigs were significantly reduced compared to hearing guinea pigs. There were no significant differences between the amplitudes when the pulse duration increased from 50 μs to 100 μs in deafened animals (\( p = 0.38 \)). However, the amplitudes in normal hearing animals presented unequal variances. Therefore, we used a Kruskal-Wallis H test and found there were significant differences (\( \chi^2 = 20.604, p = 0.002 \), and Tamhane’s T2 was then used to make pairwise comparisons among the means. In normal hearing animals, we found that oABR amplitudes were significantly different when pulse durations were between 150 μs and 50 μs (\( p = 0.039 \)), as well as between 80 μs and 50 μs (\( p = 0.015 \)). Even though, the variation tendencies of amplitudes of the normal hearing and deafened guinea pigs were similar. At the same output power of the diode laser, amplitudes increased as pulse durations were shortened. With a pulse duration of 50 μs, maximal amplitudes were 2.29 ± 0.13 μV and 0.88 ± 0.22 μV in normal hearing and deafened animals, respectively.
Fig. 4. a. Energy thresholds of normal hearing (gray curve) and deafened (red curve) animals. Deafened animals require larger stimulation energies at similar pulse durations compared to normal hearing animals. Fig. 4. b Amplitudes of waves III of oABRs at various pulse durations in normal hearing and deafened animals at the same power output of laser device. While amplitudes increased with a shortening of pulse duration in both groups, deafened animals had decreased amplitudes at all pulse durations compared to normal hearing control animals.
Deafening damages outer and inner hair cells

To determine any morphological differences between normal hearing and deafened guinea pigs, we immunostained with myosin VIIa and TuJ1 antibody. We found strong myosin VIIa labeling in the inner (IHC) and outer hair cells (OHCs) of hearing guinea pigs, but only weak labeling in the IHCs and no labeling in the OHCs of deafened guinea pigs in basal and middle turns (Fig. 5). Both IHCs and OHCs were labelled by myosin VIIa with hyper-fluorescence in the apical turn of both the normal hearing and deafened animals (Fig. 5). In addition, TuJ1 expression patterns in SGNs of both normal hearing and deafened guinea pigs were similar at one-week post-deafening (Fig. 5). These results demonstrate that after kanamycin sulfate and furosemide treatment, the OHCs of the basal and middle turns are primarily damaged, and that IHCs might also be damaged except for the apical turn.
Fig. 5. Immunofluorescence staining of SGNs (Fig. 5a presented the basal turn, Fig. 5b presented the middle and apical turn). Tuj1 labeling (green, star) was similar between normal hearing and deafened animals. In basal turn of the cochlea, myosin VIIa labeling (red) in normal animals was strong in both inner (D, arrow) and outer hair cells (D, asterisk) but weak in only the inner hair cells (H, arrow) of deafened animals (a). In middle turn, inner hair cells became hyperfluorescence in deafened animals as the inner hair cells in normal hearing animals (K and I, red arrow), the outer hair cells were still dim (I, red asterisk). Both inner and outer hair cells labeled by myosin VIIa in apical turn in deafened animals (L).

Deafening damages mitochondria and disrupts myelination of SGNs

We used TEM to investigate the ultrastructural differences in SGNs between normal hearing and deafened guinea pigs at one-week post-deafening. In normal hearing animals, the mitochondrion in SGNs were clear, with defined cristae, free of swelling or vacuolation, the normal compact structure of the myelin sheath were surrounded both the soma and axons of SGNs in normal hearing animals, and the lamellae were clear and compactly arranged (Fig. 6(a), 6(b), 6(g)). On the contrary, in animals one-week post-deafening, we observed swollen and vacuolar mitochondria in type I cells, and signs of demyelination were found on both the soma and axons of SGNs in basal turns (Fig. 6(e), 6(f)). The myelin lamellae were loose and separated from the cytomembrane in both basal and middle turns (Fig. 6(c), 6(d), 6(h)). But those variation could not be found in the apical turns of one-week post-deafening animals (Fig. 6(i)). These results suggest a degeneration of SGNs in basal and part of middle turns in one-week post-deafening animals. Of the few type II cells were observed in the present study, no differences in type II cells could be presented between normal hearing and deafened animals.
Fig. 6. TEM images of SGN (type I cell) ultrastructure of both normal hearing and deafened guinea pigs. Normal hearing guinea pigs had normal myelination around SGC axons (a, basal turn) and clear mitochondria, free of swelling or vacuolation (b, basal turn, g, middle turn). Deafening produced myelination abnormalities (c and d, green arrows), including loose lamellae that separated from the cytomembrane, and also led to swollen and vacuolar mitochondria (e and f, red asterisks) in the basal turn. In the middle turn, deafening induced myelination abnormalities, such as the partially uncompacted of myelin (h, red arrows), but no changes were presented on mitochondria (h, green asterisks). In apical turn, no differences could be found between deafened animals (i) and normal hearing animals. Magnification, a: × 3,000; c: × 5,000; b, d, g, h, i: × 20,000, e, f: × 30,000.

Discussion

CIs, which are considered the most successful neural prostheses, bypass the non-functional cochlear hair cells with an electrode array that stimulates SGNs, restoring partial hearing in severely-to-profoundly deaf individuals [11]. However, because the electrical fields spread widely along the modiolus and interact with which generated by the nearby electrodes broadly and overlap with each other, speech recognition and frequency resolution in patients with CIs, especially in noisy environments, are limited. Compared to electrical stimulation, optical stimulation of the cochlea has attracted attention due to its high spatial precision, superior...
resolution, non-overlapping stimulation, with few stimulation artifacts or electrochemical interactions between the stimulation source and the tissue [2, 3, 20].

Previous studies on infrared laser auditory stimulation focused on cochlear functions by recording CAPs [3, 8, 11, 14, 21, 22], and only a few studies measured oABRs evoked by optical stimulation in normal hearing animals [15, 23]. The present study demonstrates that infrared laser pulses with wavelengths of 1850 nm could stimulate SGNs to evoke stable oABRs in both normal hearing and deafened guinea pigs. The wave patterns of both aABRs, and oABRs in normal hearing and deafened guinea pigs were similar and reproducible. Furthermore, the prolonged latency and diminished amplitudes are associated with attenuated stimulation energy in both normal hearing and deafened animals.

The combination of kanamycin sulfate and furosemide administration is a well-established procedure for damaging hair cells in animal models [24–26]. In this study, guinea pigs were used because the cochleae are mature at birth, similar to humans, making them a suitable animal model for CI research over other rodents. We measured aABRs to estimate the hearing threshold before and post-deafening. Hearing loss was defined as a shift of 40 dB SPL post-deafening [18]. Severe sensorineural hearing loss means that no aABR waves are induced at 93 dB SPL in both ears [19]. In the present study, we recorded aABRs at four frequencies: 8, 16, 24, and 32 kHz. In deafened guinea pigs, the average threshold at 16, 24, and 32 kHz exceeded 93 dB SPL in both ears; at 8 kHz, the thresholds were 83.3 dB SPL in the left ear and 84.6 dB SPL in the right ear. It indicated that those deafened animals might retained some residual hearing at the frequencies below 8 kHz, and the hair cells located in the range of frequencies below 8 kHz were still functional. Those functional hair cells might involve in generating oABR according to the reported “photomechanical reactions” [9] and “optophonic effect” [27, 28]. On the basis of those theories, a sound wave or stress wave could be generated by the laser stimulation and propagated in the lymphatic fluid to vibrate the basilar membrane and stimulated the residual functional hair cells in low frequencies. Nevertheless, for wavelength of 1850 nm, stress wave confinement to occur when the pulse duration should be shorter than 500 ns [29], which is orders of magnitudes shorter than the pulse duration used in the recent research. In this case, it was improbable that the laser induced liquid waves can vibrate the basilar membrane and stimulate the hair cells. On the other hand, it has been demonstrated that infrared laser pulses result in a focused pressure wave in front of the optical fiber [27], the optical energy does not spread or scatter in the tissue, the light was absorbed by the volume of tissue/fluids directly in the optical path [30]. In the current study, since we placed the laser fiber close to the round window, only SGNs in the basal turn of cochlear adjacent to the round window were stimulated. According to the frequency maps in the basilar membrane [31], in guinea pigs, 8 kHz frequencies are mapped at about 68% of the distance from the apex of the cochlea, which is located in the upper basal turn, near the middle turn of the cochlea; this position is far from the stimulation location in the current study. The results showed that the oABRs could be recorded only when the optical fiber pointed to the spiral ganglion neurons region. No detectable oABRs could be recorded when the optical fiber pointed to the basilar membrane or any other directions (Fig. 1(b), 1(c), 1(d)). The results indicated that it is improbable that the residual hair cells in middle turn or apex involved in generating oABR.

Our results further demonstrated that one week after kanamycin sulfate and furosemide treatment, most OHCs were destroyed and IHCs were also damaged in the basal turn. There were no obvious morphological differences between the SGNs of normal hearing and deafened guinea pigs based on Tuj1 staining. However, electron microscopy revealed severe degeneration of type I SGNs, but no differences in type II SGNs were observed between normal hearing and deafened animals. Since 90-95% of SGNs are type I cells that are myelinated, while the remaining are unmyelinated type II cells [32], likely accounts for the predominance of type I cells damaged during chemical deafening. Indeed, in the current study, swollen and vacuolar mitochondria could be identified, and myelin abnormalities were
identified on both the soma and axons of SGNs in animals one-week post-deafening, strongly suggesting that these are type I cells degeneration. Nonetheless, previous studies found that SGNs degenerate following hair cell loss up to 15 days post-deafening [33, 34]. The interpretation may be due to the fact that previous work focused on count of SGNs number, efferent and afferent fibers, rather than focused on the ultrastructural alterations of SGNs. The degeneration in ultrastructures we observed may attenuate the functions of SGNs. Consequently, our results suggest that there are a reduced number of SGNs that can be simultaneously depolarized in response to the optical stimuli. Similar to the acoustical stimulation, the number of SGNs that are simultaneously depolarized in response to optical stimuli determine the amplitude and energy thresholds of the oABR [15].

The energy thresholds and amplitudes of oABRs correlated not only with the number of SGNs that simultaneously depolarized in response to optical stimuli, but also with the laser pulse durations. The variation of oABR energy threshold was associated with the change of pulse duration both in normal hearing and deafened guinea pigs. The energy thresholds of oABRs were elevated as the pulse duration increase. In other words, the radiant exposure required to stimulate SGNs increased as the pulse duration lengthened [14]. However, for short pulse durations (50–100 μs), differences were not statistically significant. When the pulse duration increased to 150 μs, differences became statistically significant compared to the shorter pulse duration. It has been hypothesized that optical stimulation of neural responses occurs via a photothermal mechanism [9]; the amount of energy absorbed by the tissue governs the temperature increase subsequent to the light absorption. Shorter pulse durations appear to be more efficient in evoking an action potential when stimulating the SGNs of the cochlea, the transience of the optical pulse was the important factor for infrared neural stimulation of the cochlea and not the total energy [2, 15, 35].

At mid-infrared wavelengths, light is primarily absorbed by water in the tissue and converted into heat. A small, yet spatially and temporally confined increase in tissue temperature is induced by an optical-thermal mechanism [9, 15]. An increase in threshold implies more radiant exposure during the optical stimulation. Consequently, a short pulse duration, with a lower energy threshold, is a suitable laser parameter to stimulate the cochlea because of the mitigated possibility of thermal damage on SGNs. The oABR energy threshold increased significantly in the deafened animals when compared to the hearing animals, mostly likely due to a reduced number of properly functioning SGNs capable of simultaneous depolarizations in response to optical stimuli. This idea is supported by the degenerated ultrastructure of SGNs, and likely underlies the blunted amplitude responses in deafened animals.

The amplitude of oABRs was elevated slightly as the pulse duration decreased in normal hearing animals, at the same output power of the diode laser. Differences were statistically significant between pulse durations of 150 μs and 50 μs, as well as between 80 μs and 50 μs in normal hearing animals. Greater laser energy was required for longer pulse durations at the same output power. In this case, to attain a certain amplitude of oABRs, a shorter pulse duration with a lower radiant energy delivered to the target structures, such as SGNs, would reduce the possibility of thermal damage by the optical stimulation. Regardless, the tendencies of amplitudes were similar between both the normal hearing and deafened guinea pigs. Moreover, we found that maximal amplitudes evoked by the pulse duration of 50 μs were greatly reduced in both normal hearing and deafened animals. Although the amplitudes evoked by various pulse durations were fairly different in deafened animals, no significant differences were found between amplitudes at various pulse durations post-deafening. Further studies are necessary to clarify whether the shorter pulse duration presented higher amplitudes under the same stimulus level, as the SGNs degenerated when the animals were deafened following kanamycin sulfate and furosemide treatment.

The energy delivered to the target tissue depends on the distance between the end of the optical fiber and the SGNs, along with any light absorber(s) or scatterer(s) in the optical path.
In the current study, radiant energy were measured at the end of the optical fiber in air. The penetration depth in water of 1850 nm wavelength is about 1.016 mm [37]. Penetration depth is defined as the distance over which the incident energy is reduced to 1/e, or ~37% of its original value. Since most hair cells in the basal turn of the cochlea were damaged or dead, even if functional hair cells are spared in the middle or apical turn, the optical fiber placement and rapid distance-dependent decay of the light stimulation suggests that these surviving cells are unlikely to underlie the evoked oABRs. Furthermore, no optical energy was delivered to the contralateral side of cochlea [30].

In summary, we found that kanamycin sulfate and furosemide treatment damages hair cells in the basal turn of the cochlea because aABRs were diminished and aberrant myosin VIIa staining. However, oABR energy thresholds elevating and amplitudes decreasing in deafened animals are likely due to the degeneration of SGNs, which would lead to a smaller population of SGNs that are able to respond to infrared laser stimuli.

Conclusions

Infrared laser pulses are able to stimulate SGNs to evoke oABRs in both normal and deafened guinea pigs. Deafening increases energy thresholds while reducing amplitudes, likely due to the degeneration of SGNs, which induced a weakened response, not attributable to the hair cells. In addition, to stimulate the cochlea in both normal and deafened animals, short pulse durations are more suitable as laser parameters.

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