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Thompson, A. C., Fallon, J. B., Wise, A. K., Wade, S. A., Shepherd, R. K., & Stoddart, P. R. (2015). Infrared neural stimulation fails to evoke neural activity in the deaf guinea pig cochlea. *Hearing Research*, 324, 46-53.

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DOI: 10.1016/j.heares.2015.03.005

# Infrared neural stimulation fails to evoke neural activity in the deaf guinea pig cochlea

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## Abstract

At present there is some debate as to the processes by which infrared neural stimulation (INS) activates neurons in the cochlea, as the lasers used for INS can potentially generate a range of secondary stimuli e.g. an acoustic stimulus is produced when the light is absorbed by water. To clarify whether INS in the cochlea requires functioning hair cells and to explore the potential relevance to cochlear implants, experiments using INS were performed in the cochleae of both normal hearing and profoundly deaf guinea pigs. A response to laser stimulation was readily evoked in normal hearing cochlea. However, no response was evoked in any profoundly deaf cochleae, for either acute or chronic deafening, contrary to previous work where a response was observed after acute deafening with ototoxic drugs. A neural response to electrical stimulation was readily evoked in all cochleae after deafening. The absence of a response from optical stimuli in profoundly deaf cochleae suggests that

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the response from INS in the cochlea is hair cell mediated.

*Keywords:* infrared neural stimulation, INS, cochlear implant, optical stimulation, optoacoustic

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## 1. Introduction

Despite the great success of the cochlear implant, used by over 300,000 patients worldwide (NIH, 2014), many patients are limited in their ability to understand speech in noisy environments or appreciate music (Friesen et al., 2001). This limitation appears to be primarily due to spreading of electrical current in the cochlea (Friesen et al., 2001), which reduces the effective number of independent channels and thus, spectral information that can be delivered. Present research is exploring a range of approaches to improve delivery of spectral information, including techniques to reduce the spread of current from electrodes (George et al., 2014) and the use of optical stimulation techniques (Hernandez et al., 2014; Izzo et al., 2006).

Infrared neural stimulation (INS) is a proposed alternative to electrical stimulation using micro- to milli-second duration pulses of infrared light to activate neurons (Richter et al., 2011; Chernov and Roe, 2014). Compared to traditional electrical stimulation, reports in the sciatic nerve have suggested that INS can be more spatially localised (Wells et al., 2007) and, unlike optogenetic and caged molecule techniques (Kramer et al., 2009), modification of the target tissue is not required (Richter et al., 2011). INS has been demonstrated *in vivo* in a number of targets, including: peripheral nerves (Wells et al., 2005b), visual cortex (Cayce et al., 2014), embryonic heart (Jenkins et al., 2010) and the cochlea (Izzo et al., 2006). The mechanism of INS has been a topic of much discussion in the literature (Brown et al., 2013; Shapiro et al., 2012; Albert et al., 2012). The potential mechanisms that have been identified for INS include a change in the cell membrane capacitance in response to rapid heating (Shapiro et al., 2012) and activation of TRPV heat sensitive ion channels (Albert et al., 2012).

Electrical stimulation in the cochlea targets the primary auditory neurons known as spiral ganglion neurons (SGNs). Applying INS to the cochlea has been an area of particular interest, as the potential for an improvement in the spatial selectivity of INS over electrical stimulation may be advantageous for use in cochlear implants (Thompson et al., 2013b). Previous work has shown that INS activates spatial regions similar to acoustic tones, suggesting im-

proved spatial localisation compared to electrical stimulation. Furthermore, excitation is largely restricted to SGNs in the beam path. However, much  
35 excitation appears to be on side of the cochlea opposite the optical fibre, where both SGNs and hair cells would be exposed to the light. Rather than those directly in front of the optical fibre (Moreno et al., 2011), where only SGNs were exposed. It should also be noted that radiant exposure thresholds for neural activation reported for INS in the cochlea ( $3 - 20 \text{ mJ.cm}^{-2}$   
40 (Izzo et al., 2006; Richter et al., 2011, 2008; Thompson et al., 2013a)) are much lower than those reported for other neural targets, where thresholds of  $320 - 710 \text{ mJ.cm}^2$  are commonly required (Wells et al., 2005a; Teudt et al., 2007), suggesting differences in the mechanisms associated with evoking the neural response.

45 Many reports of INS in the cochlea use normal hearing animals (Matic et al., 2013; Izzo et al., 2007). Some previous experimental reports discuss the ability of INS to evoke neural activity in animals acutely deafened with ototoxic aminoglycosides (Izzo et al., 2006; Richter et al., 2008), which disable and eliminate the hair cells. This suggests that this INS-evoked neural  
50 activity is a direct interaction between the laser and the SGNs. However, the deafening techniques used in these reports have shown varying levels of hearing impairment. Furthermore, INS in chronically deaf animals could only evoke a response when one could also be evoked with an acoustic stimulus (Richter et al., 2008).

55 This has led to some doubt about the stimulation mechanism in cochlear studies, which questions as to whether INS directly activated the SGNs, or whether SGN activation occurred as a result of hair cell mediated responses (Schultz et al., 2014). This is an important distinction for the application of INS with a cochlear implant where recipients typically have severe to  
60 profound deafness with few (if any) functional hair cells remaining. Laser sources used for INS have been shown to generate an acoustic click from rapid expansion of heated water, resulting in an optoacoustic mediated response (Teudt et al., 2011). Optoacoustic generation of sound has been shown to be applicable to many different wavelengths, including those used by INS,  
65 and appears to be driven by water or haemoglobin absorption (Schultz et al., 2012; Rettenmaier et al., 2014). Therefore, if the deafening process is not complete and functional hair cells remain, the generation of an acoustic click during INS may lead to a response mediated by activation of residual hair cells by this acoustic artefact. Furthermore, a recent report of INS in the rat  
70 cochlear nucleus, an auditory structure remote from hair cells of the cochlea,

found no response after deafening that was achieved by cutting the auditory nerve at the internal auditory meatus (Verma et al., 2014).

Given the conflicting results described above, further investigation is required to determine the mechanism of INS in the cochlea and to clarify the potential benefits of the technique. In this paper, results of INS in severely-profoundly deaf guinea pig cochleae are reported. Both acute and chronic deafening techniques have been used, in order to investigate the potential role of the deafening procedure in determining the outcome of INS experiments.

## 2. Methods

### 2.1. Animal Preparation

All experimental procedures involving animals were performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and with the guidelines laid down by the National Institutes of Health in the USA regarding the care and use of animals for experimental procedures. These procedures were approved by the Royal Victorian Eye and Ear Hospital Animal Research and Ethics Committee.

Four ( $n = 4$ ) young adult male or female pigmented guinea pigs were used to collect data for the experimental studies presented in this paper. In two animals both cochleae were tested with INS, while the other two were only tested on one side, giving a total of six cochlea examined ( $n_{\text{cochlea}} = 6$ ). Two were used for acutely deafened experiments, while two were chronically deafened four weeks before stimulation experiments. All acutely deafened animals were first tested as control normal hearing animals, with INS experiments performed before the deafening process was carried out. In chronically deafened animals INS experiments were carried out on both cochleae.

Anaesthesia was induced with an intramuscular delivery of ketamine ( $60 \text{ mg.kg}^{-1}$ ) and xylazine ( $4 \text{ mg.kg}^{-1}$ ) and was maintained with top-up doses of ketamine ( $40 \text{ mg.kg}^{-1}$ ) and xylazine ( $4 \text{ mg.kg}^{-1}$ ), administered at one third to one sixth of the induction volume every 40 – 50 min, or if a toe pinch withdrawal reflex was present. The animal was kept on a thermostatic heating pad at  $38 \text{ }^\circ\text{C}$  to maintain body temperature in the normal range.

Animals were prepared for surgery by shaving the area near the neck and skull and then injecting a local anaesthetic (lignocaine, 0.1 ml) at the incision site. A post-auricular incision was made and the temporalis muscle retracted, exposing the tympanic bulla. The dorsal region of the bulla was then drilled with a 2 mm cutting burr to expose the cochlea. A cochleostomy was drilled

into the otic capsule of the basal turn using a diamond burr to thin the cochlea wall. After clearing the bone debris, the endosteum was perforated to expose the scala tympani and the modiolus (Figure 1). In some animals  
110 a cochleostomy was performed in a more apical turn to assess whether the spiral ganglion neuron population at this location responded differently to those located in the basal turn.

At the conclusion of the experiment, the animals were sacrificed with an overdose of anaesthetic sodium pentobarbital ( $150 \text{ mg.kg}^{-1}$ ) and intracardially perfused with formalin fixative. Cochleae were collected for histological  
115 analysis.

## 2.2. Deafening

To prevent hair cell mediated electrophonic or optoacoustic (Teudt et al., 2011) activity from being confused with a direct neural interaction with the  
120 laser, animals were deafened using procedures previously shown to produce acute hearing loss (Hardie and Shepherd, 1999) or chronic, bilaterally symmetric, hearing loss (Landry et al., 2011; Kral et al., 2002). These procedures differ from those commonly used in previously reported cochlear INS studies.

Acute deafening was performed unilaterally by opening both the oval  
125 and round windows and gently aspirating neomycin ( $10 \text{ mg.ml}^{-1}$ ) through the cochlea from the round window to oval window. Aspiration through the cochlea ensured exposure of the apical region of the cochlea to neomycin, which may not be thoroughly exposed without aspirating. The procedure was repeated at least three times to ensure thorough ototoxin exposure. The  
130 acute deafening procedure was performed after taking normal hearing control recordings with INS and electrical stimulation.

Neomycin has been shown to block calcium channels (Zhou et al., 1996) and calcium appears to be involved in the response attributed to INS (Ditami et al., 2011; Paviolo et al., 2013; Lumbreras et al., 2014). Therefore it  
135 is possible that neomycin may interfere with INS and block a response from being observed in the SGNs of acutely deafened animals. In chronically deaf animals, ototoxic drugs should have cleared by the time of the experiment. To investigate if neomycin may block a response, the ability of INS to evoke a response in chronically deaf animals was also investigated.

Systematic chronic deafening was performed four weeks before experimentation, under gaseous anaesthesia ( $1\text{--}2\%$  isoflurane in  $\text{O}_2$ ,  $1 \text{ L.min}^{-1}$ )  
140 using a combination of frusemide ( $130 \text{ mg.kg}^{-1}$ , administered intravenously) and kanamycin ( $420 \text{ mg.kg}^{-1}$ , administered subcutaneously) (Landry et al.,

2011). The hearing status of chronically deafened animals was assessed under  
145 anaesthesia with click stimuli one week before deafening and one week after  
deafening to confirm normal hearing status and then confirm hearing loss.  
The animal was defined as severe-profoundly deaf if the hearing threshold  
increased by  $\geq 50$  dB. After the experiment was terminated, histological  
analysis of the exposed cochlea was performed to confirm hair cells loss.

### 150 *2.3. Infrared and Electrical Stimulation*

An Aculight Renoir laser (Lockheed Martin Aculight Corp., Bothell, WA)  
provided the optical stimulus. This laser has been used in a number of  
previous INS studies (e.g. (Richter et al., 2008)). The laser was coupled to  
200  $\mu\text{m}$  core diameter fibre (NA = 0.22) that was attached to a quartz glass  
155 micropipette. This micropipette was mounted on a 2662 Micropositioner  
(Kopf, USA) which controlled the insertion of the optical fibre in the cochlea.  
Pulse energy at the fibre tip was measured in air with a Coherent Fieldmaster  
and Coherent Model LM-10 detector head at the beginning and end of each  
experiment. Unless otherwise stated, a wavelength of 1869 nm was used,  
160 giving an optical penetration depth ( $1/\mu_a$ ) in water of approximately 500  $\mu\text{m}$   
(Thompson et al., 2012). Pulse durations of 100  $\mu\text{s}$  were typically used, with a  
repetition rate of 20 Hz and energy of up to 120  $\mu\text{J}$  giving a maximum radiant  
exposure of 382  $\text{mJ}\cdot\text{cm}^{-2}$  at the fibre tip, similar to parameters successfully  
used for cochlear INS in the literature (e.g. Ref (Moreno et al., 2011)). A  
165 range of stimulus durations from 100  $\mu\text{s}$  to 10 ms were used, with the longer  
pulses providing correspondingly greater pulse energies similar to those used  
for other INS targets (e.g. the sciatic nerve (Wells et al., 2007)). For pulses  
of 1 ms or longer, the repetition rate was reduced to 2 Hz, to minimise the  
potential for heat-induced damage to the cochlea (Thompson et al., 2013b).

170 Monopolar electrical stimuli were delivered by a platinum ball electrode,  
placed inside the cochlea through the cochleostomy and stainless steel return  
electrode placed in the neck. Electrical stimuli were presented with single  
biphasic anodic leading phase square current pulses, either 25  $\mu\text{s}$ /phase with  
8  $\mu\text{s}$  interphase gap, or 100  $\mu\text{s}$ /phase with 50  $\mu\text{s}$  interphase gap at a repe-  
175 tition rate of 20 Hz. Before beginning INS stimulation experiments, normal  
cochlea function was determined by recording auditory brainstem responses  
to electrical or acoustic stimuli.

#### 2.4. ABR Recordings

The response of the cochlea and auditory system was assessed by recording the auditory brainstem response (ABR) to acoustic (aABR), electrical (eABR) and optical stimulation (oABR), using standard techniques (Landry et al., 2011; Coco et al., 2007). All recordings were performed in an electrically and acoustically isolated Faraday room. Three stainless steel electrodes were used (vertex positive, neck negative and thorax ground), to differentially recorded the signal. ABRs were averaged across 100 trials, presented at a rate of 20 Hz, and at least two sets of recordings were obtained at each stimulus intensity level. ABRs were recorded with Igor Pro software (Wavemetrics, USA) via a NI USB-6251 data acquisition device (National Instruments, USA) (100 kHz sampling). After acquisition the first  $\sim 1$  ms of the signal was discarded to remove any electrical artefact and the resultant waveform was digitally filtered with a 300 – 3000 Hz bandpass filter (Butterworth, second order).

#### 2.5. Histological Preparation

The osseous spiral lamina was fractured by the optical fibre at the conclusion of the experiment in order to confirm the location and orientation of the fibre during the stimulation experiments (1, arrow at circle). Following the acute experiment the animals were euthanised ( $150 \text{ mg.kg}^{-1}$  lethabarb; intraperitoneal) and transcardially perfused with 0.9% NaCl ( $37^\circ\text{C}$ ) followed by 10% Neutral Buffered Formalin (10% NBF;  $4^\circ\text{C}$ ). The cochleae were removed and the round and oval windows were opened before post-fixation in 10% NBF. The cochleae were then placed in 10% ethylenediamine tetraacetic acid (EDTA) in PBS at room temperature for decalcification. Following decalcification the cochleae were cryo-protected in 15% and then 30% sucrose and then embedded in Tissue-Tek O.C.T. cryosectioning compound (Sakura, Japan) and frozen, as described previously (Wise et al., 2011). Cochleae were sectioned in  $12 \mu\text{m}$  slices using a CM 1900 UV cryostat (Leica, Germany) and mounted onto Superfrost-Plus slides (Menzel-Gläser, Braunschweig, Germany). A representative series of cochlear sections were stained with Mayer's haemotoxylin and Putt's eosin (H&E) for histological examination.

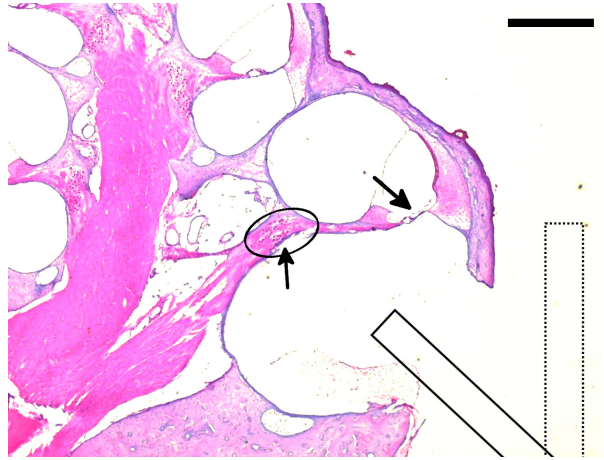


Figure 1: Histological image of a mid-modiolar cochlear section from a normal hearing animal indicating the cochleostomy and the target spiral ganglion neurons (oval). The organ of Corti containing the intact sensory hair cells (arrow) is intact in this example. The osseous spiral lamina was fractured by the optical fibre (arrow at circle). Solid lines represent optical fibre position when targeting the SGNs and dashed lines show optical fibre next to, but not pointing to the cochlea. Scale bar = 500  $\mu\text{m}$ .

### 210 3. Results

#### 3.1. Normal hearing and acutely deafened animals

Infrared stimulation was attempted in two animals, acutely deafened with neomycin. The first animal was initially exposed to INS before deafening as a normal hearing control. As the pulses used by INS lasers are capable of generating sound in a humid atmosphere or water (Teudt et al., 2011), the pure optoacoustic response of the normal hearing cochlea was assessed before performing the cochleostomy. The optoacoustic response was found by placing the optical fibre next to the intact cochlea, but not oriented towards the SGNs (Figure 1, dashed line). The ABR recording (Figure 2) shows a clear response to the optoacoustic stimulus generated by the laser pulse. As expected, the ABR recording to the optoacoustic stimulus shows similar waveform shape to that in response to an acoustic click (Fig 3).

After opening the cochlea, the fibre was inserted through the cochleostomy and directed towards the SGNs (Fig 1, solid line). Accurate positioning of the fibre was confirmed by retracting the fibre after attempting optical stimulation and damaging the osseous spiral lamina with the optical fibre at the

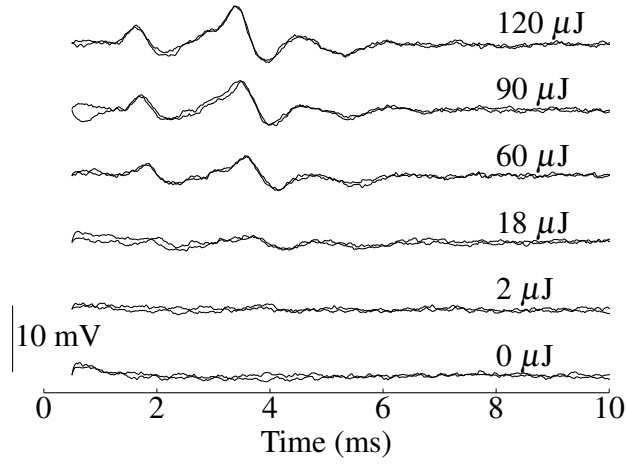


Figure 2: ABR recording from normal hearing cochlea evoked by the Aculight INS laser ( $t_{\text{pulse}} = 100 \mu\text{s}$ ) next to normal hearing cochlea but not directed towards the SGNs (dashed lines Fig 1).

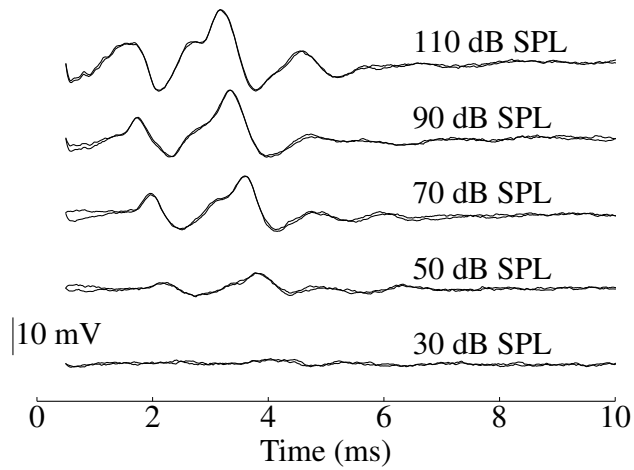


Figure 3: An acoustically-evoked aABR recorded from a normal hearing cochlea.

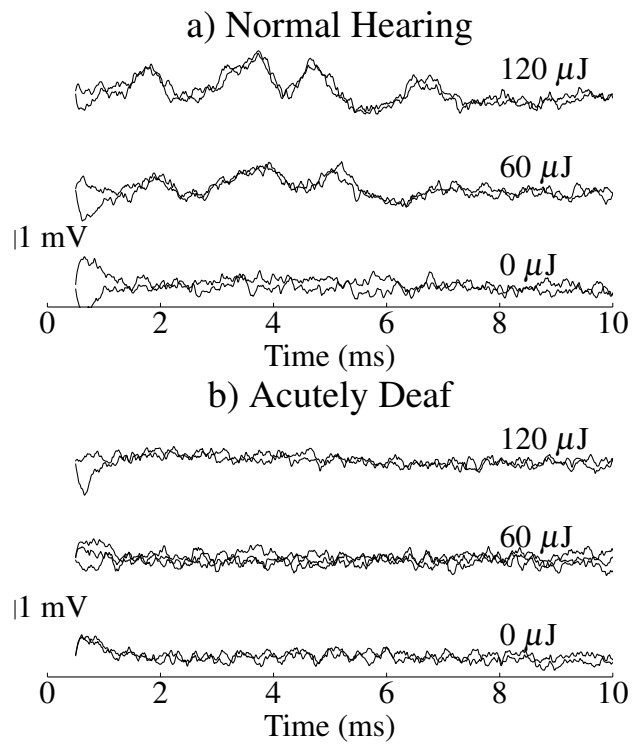


Figure 4: oABR recording from a) normal hearing and b) acutely deaf cochlea evoked by the Aculight INS laser ( $t_{\text{pulse}} = 100 \mu\text{s}$ ) with the fibre aimed at the SGNs inside the cochlea.

Animal		1	2
Hearing Status			
Normal Hearing	aABR	50 dB	60 dB
	eABR	NM	975 mA
	oABR	30 $\mu$ J	NR
Post-Deafening	eABR	625 mA	950 mA
	oABR	NR	NR

Table 1: Summary of thresholds found in normal hearing and acutely deaf animals. NR indicates no response could be evoked, NM indicates that this measurement was not made.

conclusion of the experiment. Histological images of this cochlea demonstrates that the SGNs were on the optical axis of the optical fibre (Fig 1).

Figure 4a shows the response of the infrared laser targeting the SGNs before and after deafening with neomycin. A clear response can be seen in the normal hearing case, using  $t_{\text{pulse}} = 100 \mu\text{s}$  and a pulse energy of  $120 \mu\text{J}$  evoking a response of 2.6 mV, smaller than the response found before opening the cochlea. After deafening, no response could be observed with the fibre in the same position and for pulse energies up to the maximum of  $120 \mu\text{J}$  (Figure 4b), while an eABR could be readily evoked with electrical stimulation (Figure 5). Optical pulse durations of up to  $t_{\text{pulse}} = 1 \text{ ms}$  with energies of 1.2 mJ were attempted without evoking a response. Thresholds for normal hearing and acutely deaf animals are summarised in Table 1.

The amplitude of responses to different stimuli is summarised in Figure 6. It shows the input/output (I/O) curves, taking the maximum peak-peak difference 2 – 5 ms post stimulus for a) the acoustic stimulus before deafening, b) response from INS and c) response from electrical stimulation after deafening. It clearly shows a stronger response is observed with acoustic stimulation compared to that evoked by INS or electrical stimulation. Acoustic stimulation gives a maximum response of 21.3 mV at 110 dB, compared to 8.3 mV at  $120 \mu\text{J}$  from the optoacoustic response with the fibre positioned next to, but not oriented towards the SGNs. A response of this amplitude would correspond to a click of approximately 50 – 60 dB, similar to the 62 dB SPL measured by Teudt et al. (2011) for a similar wavelength, pulse length and energy. The response from INS with the fibre targeting the neurons is smaller again, at just 2.6 mV at  $120 \mu\text{J}$ . A number of factors could contribute to this reduction in amplitude. These include, a reduced response to the sound generated by the laser due to opening the cochlea or a direct interaction be-

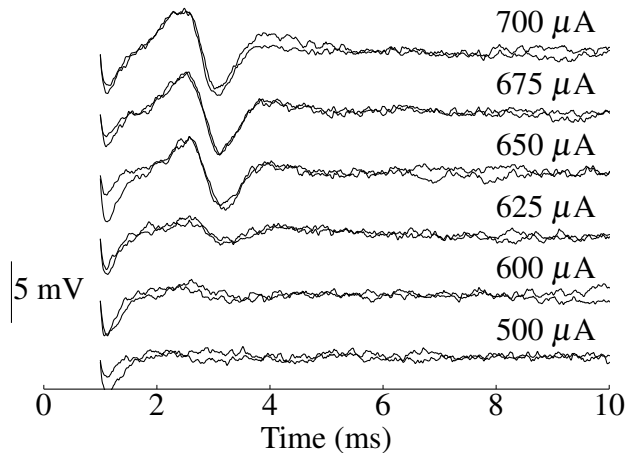


Figure 5: Typical eABR recording from electrical stimulation after deafening the cochlea with neomycin. Here monopolar stimulation was delivered via a ball electrode placed inside the cochleostomy with  $25 \mu\text{s}$  per phase.

255 between the laser and neurons. As no oABR was evoked after acute deafening, it is possible the reduction in amplitude is due to opening the cochlea.

260 Although the targeting of the optical fibre was confirmed to be accurate through subsequent histological analysis, many adjustments were made to the fibre position during the experiment in case there was a more sensitive location. The fibre was also positioned so it was touching the osseous spiral lamina, thus maximising the energy delivered at the neurons by reducing the absorption losses in the intermediate fluid (Thompson et al., 2012). No adjustments to the optical fibre position were effective in evoking a response from INS.

### 3.2. Chronically deafened animals

265 All chronically deafened animals exhibited a severe-profound hearing loss. To investigate whether the presence of neomycin may interfere with an INS mediated neural response, and to evaluate the effectiveness of INS in cases where there has been significant spiral ganglion neuron loss due to prolonged deafness, INS was performed in both cochleae of two chronically deafened animals. In two of the cochleae, the more apical turns were also exposed to INS in addition to the basal turn, as there is often a higher rate of spiral ganglion neuron survival after deafening in the apical turns (Landry et al.,

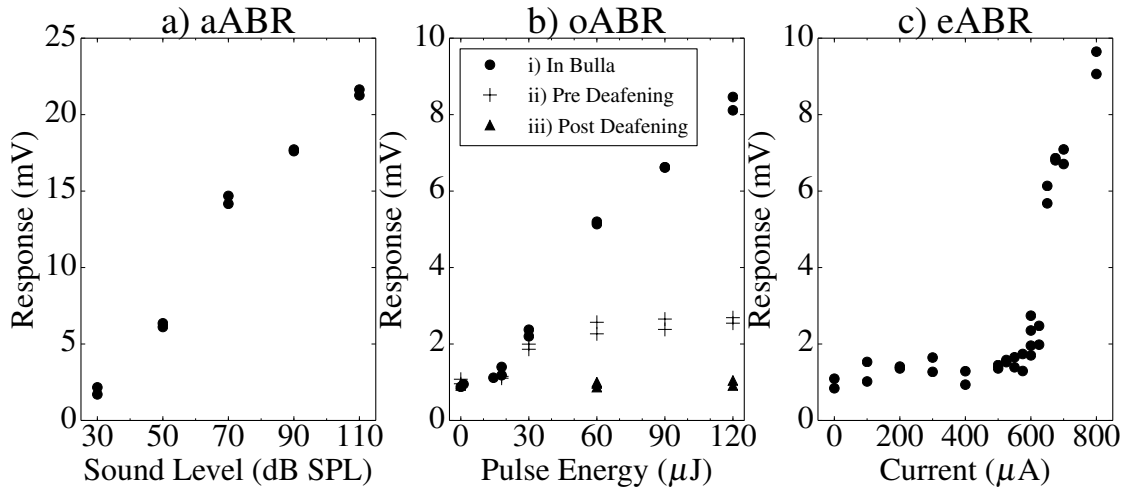


Figure 6: Input/output (I/O) plot for the maximum peak-peak difference 2 — 5 ms post stimulus for a) the acoustic stimulus before deafening, b) response from INS and c) response from electrical stimulation after deafening. Note the different response (Y axis) scale for a) compared to b and c).

Animal	3L	3R	4L	4R
eABR	1400 mA*	375 mA	300 mA	325 mA
oABR	NR	NR	NR	NR

Table 2: Summary of thresholds found in chronically deaf animals. NR indicates no response could be evoked. \*Note the different threshold for the animal three, left cochlea was due to using 25  $\mu\text{s}$ /phase, rather than the 100  $\mu\text{s}$ /phase.

2011). The higher spiral ganglion neuron count has been linked to lower thresholds for INS in the cochlea (Richter et al., 2008).

275 INS with pulse durations of 100  $\mu\text{s}$  to 10 ms, with corresponding maximum pulse energies of 120  $\mu\text{J}$  to 12 mJ were used. No response to INS was evoked in any cochlea at any pulse energy or position. An example of the null response to a 100  $\mu\text{s}$  duration pulse with  $E_{\text{pulse}} = 120 \mu\text{J}$  is shown in Figure 7. An eABR was evoked in all cochleae in response to electrical stimulation. Thresholds for stimulation in chronically deaf animals are summarised in Table 2. Similarly to the acutely deafened animals, a number of optical fibre positions were attempted to ensure that the SGNs were exposed to INS. Histological analysis of these cochleae confirmed the absence of hair cells in the organ of Corti (Fig 8).

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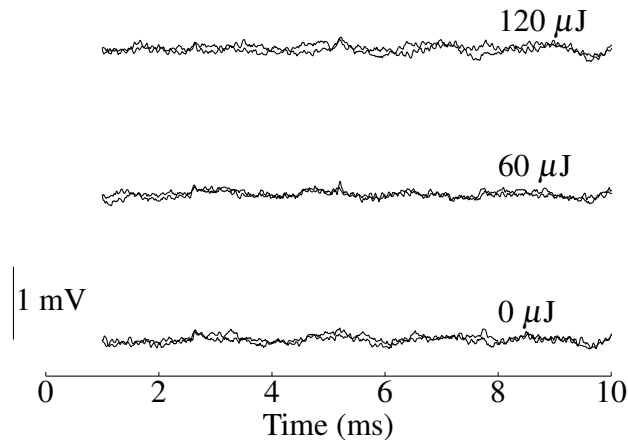


Figure 7: Typical oABR recording from INS in chronically deafened animals. Here a  $100 \mu\text{s}$  duration pulse was used, over an intensity range of  $0 - 120 \mu\text{J}$  without evoking a neural response. The optical fibre was aimed at the SGNs and many different optical fibre position were attempted.

#### 285 4. Discussion

This study investigated INS in normal hearing, acute and chronically deaf guinea pig cochleae. An oABR could be evoked in a normal hearing cochlea, but no oABRs were observed in deafened cochleae. An eABR could be readily evoked with electrical stimulation in all cochleae post-deafening  
 290 indicating that the SGNs were functionally viable. The primary difference between this and previous work appears to arise in the deafening protocol. Here, animals treated with ototoxic drugs were severe-profoundly deaf, while animals in many previous studies have a level of residual hearing (Richter et al., 2011; Moreno et al., 2011; Richter et al., 2008), or were not deafened at  
 295 all (Matic et al., 2013; Izzo et al., 2007). Therefore, the null results presented here suggest that the previously reported instances of INS in the cochlea may be mediated by hair cells, rather than by a direct interaction with the SGNs.

Chronically deaf animals typically show pathological changes in the SGNs, with both cell death and loss of peripheral processes (Xu et al., 1993; Shepherd and Hardie, 2001). These changes result in higher thresholds for electrical stimulation and reductions in response amplitude of  $> 70 \%$  (Shepherd and Hardie, 2001). Previous cochlear INS studies have assessed chronically  
 300 deaf animals where the cochleae were treated with high levels of neomycin

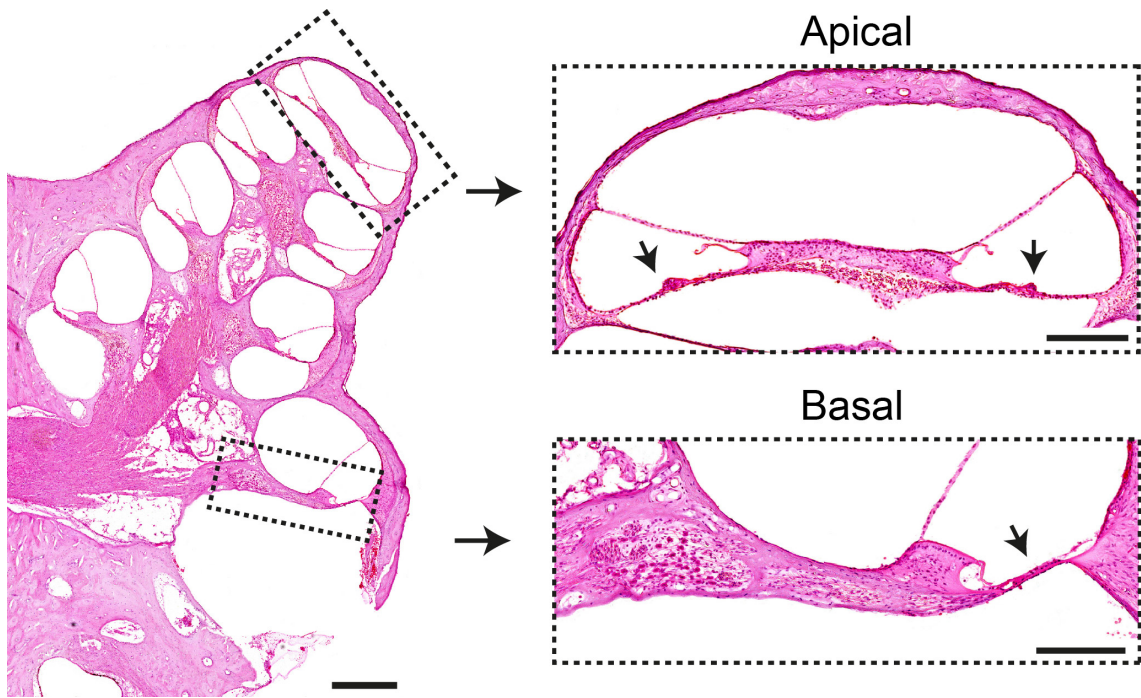


Figure 8: Histological image of a mid modiolar cochlear section from a chronically deaf animal indicating the cochleostomy, with inserts highlighting the absence of hair cells in the organ of Corti (arrow). Scale bar = 500  $\mu\text{m}$  for main figure and 200  $\mu\text{m}$  for inserts.

(Richter et al., 2008). In that case, if the cochlea showed no response to  
305 infrared stimulus, it was also found to give no response to acoustic or electrical stimuli (Richter et al., 2008). In the present work, a clear electrical response was evoked in all chronically deafened animals, but no response to INS or acoustic stimulation was evoked. This implies that the neurons were functional but not excitable with INS.

#### 310 *4.1. Optoacoustic Responses*

Given that the response to infrared laser stimulation disappeared after deafening in this work, it is plausible that the response attributed to INS in prior reports in the cochlea is due to the optoacoustic effect as previously reported by Teudt et al. (2011). A purely acoustic effect would also  
315 correlate well with a recent report of INS targeting the rat cochlea nucleus, which reported no response after deafening by cutting the auditory nerve at the internal auditory meatus (Verma et al., 2014). Cutting the auditory nerve prevented transmission of acoustically evoked responses generated in the cochlea from reaching the the recording site in the auditory brainstem,  
320 while still allowing responses evoked in the cochlea nucleus to be transmitted to the recording site, thus preventing acoustically evoked neural activity from reaching the nerves being stimulated and recorded.

A similar result has been seen using nanosecond duration laser pulses with wavelengths other than those typically used for INS (420 – 2150 nm) (Schultz  
325 et al., 2012; Wenzel et al., 2009), with a clear response observed in normal hearing animals and no response after acute deafening with neomycin. The absence of a response after deafening is a strong indication of an optoacoustically mediated response to the nanosecond duration laser pulses.

However, a purely optoacoustic mechanism is not consistent with all previously reported results of INS in the cochlea. For example, Moreno et al.  
330 (2011) showed excitation of SGNs was largely restricted to the area in the beam path. This would suggest a direct interaction with SGNs or hair cells, rather than an acoustic stimulation of hair cells.

#### *4.2. Other Mechanisms*

335 A number of other mechanisms may exist to explain hair cell involvement in the response attributed to INS in the cochlea without invoking a purely optoacoustic mechanism. For example, vestibular hair cells have been shown to respond to INS (Rajguru et al., 2011). Although the detailed mechanisms

in the vestibular system also remain unclear, there is evidence that the re-  
340 sponse was not due to an optoacoustic process, but rather direct stimulation  
of the hair cell; and that the mechanism may also apply to hair cells of the  
cochlea (Rajguru et al., 2011). Another possibility is that the infrared laser  
pulses may cause release of synaptic vesicles from the hair cells resulting in  
post-synaptic currents in the neurons (Liu et al., 2014). If the synapses be-  
345 tween the hair cells and spiral ganglion neurons are exposed to infrared light,  
this could drive the response observed. Furthermore, the results of Moreno  
et al. (2011) have shown excitation on the side of the cochlea spiral opposite  
to the optical fibre. In this work, little excitation was observed near the  
optical fibre, where only the SGNs would have been exposed to the light. On  
350 the side of the cochlea opposite to the optical fibre, strong excitation was  
observed. Here, both the SGNs and hair cells would have been exposed to  
the light, again suggesting an interaction with the hair cells.

Another potential mechanism comes from recent work showing that the  
motor protein in outer hair cells, prestin, can respond to rapid temperature  
355 changes such as those produced by exposure to infrared illumination (Oku-  
nade and Santos-Sacchi, 2013). If outer hair cells are exposed to infrared  
laser light, it is possible that this could mediate the response observed. Note  
that these putative mechanisms rely on some level of residual activity in the  
hair cells, which is consistent with all of the reported cases of INS in acutely  
360 deafened cochleae, as well as the absence of response in severe to profoundly  
deafened animals (both acute and chronic).

Hair cells are not present in the majority of neural targets where INS  
has been demonstrated. The radiant exposure threshold and corresponding  
temperature change required for neural activation is much greater in those  
365 cases than the for the cochlea (Thompson et al., 2013b). The tempera-  
ture increase observed (Wells et al., 2007) and predicted (Thompson et al.,  
2013b) suggests that other mechanisms such as activation of heat sensitive  
ion channels (Albert et al., 2012) changes in cell membrane capacitance may  
be responsible.

370 If hair cells respond to pulses of infrared light, it may provide a new  
technique to interrogate their function and the mechanisms of hearing. Un-  
derstanding the exact interaction between infrared laser and the cochlea will  
require further careful experimentation. Understanding the full mechanism  
may involve *in vitro* excitation of hair cells, both in isolation and in tissue  
375 slices.

### 4.3. Methodological Considerations

The number of cochleae used in this study is relatively small ( $n = 6$ ) and it could be argued that it is insufficient to make strong statements about the mechanisms of INS in the cochlea. However, an eABR could be evoked in  
380 all of the cochleae tested, thus indicating normal neural function. In each of the cochleae tested, a full range of INS pulse durations and energies were used. Furthermore, a range of fibre positions were attempted and correct positioning of the fibre was confirmed through subsequent histological analysis. Without a clear avenue to advancing the procedure for further experimenta-  
385 tion and a realistic expectation that this would change the outcome, further animal experimentation was not considered ethically justified.

## 5. Conclusion

No INS evoked oABR was observed in any deaf cochleae, while normal hearing cochlea responded to the infrared laser stimulus. These results sug-  
390 gest that to evoke an oABR from INS requires functional hair cells. Identifying the mechanism, which may be optoacoustic or some other interaction with the infrared light, will require further work. It appears unlikely that INS in an implantable device will show clinical benefits for profoundly deaf patients without significant further development. However, if the INS inter-  
395 action is mediated by hair cells, use of infrared light may provide novel ways of interrogating hair cell function and provide further insights into to the mechanisms of hearing.

## Acknowledgements

The authors thank C.-P. Richter of Northwestern University for the loan  
400 of the Aculight laser and helpful discussions. S. Irving of the Bionics Institute kindly assisted with aspects of the in vivo experiments. This work was supported by Cochlear Ltd and the Australian Research Council under grant LP120100264. The Bionics Institute acknowledges the financial support it receives from the Victorian State Government.

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