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# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>1</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>3</td>
</tr>
<tr>
<td>GLOSSARY OF ABBREVIATIONS</td>
<td>6</td>
</tr>
<tr>
<td>CHAPTER 1: INTRODUCTION</td>
<td>7</td>
</tr>
<tr>
<td>CHAPTER 2: ELECTRICAL STIMULATION OF THE COCHLEAR NUCLEUS</td>
<td>14</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>14</td>
</tr>
<tr>
<td>METHODS</td>
<td>15</td>
</tr>
<tr>
<td>RESULTS</td>
<td>20</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>31</td>
</tr>
<tr>
<td>CHAPTER 3: OPTOGENETIC STIMULATION OF THE COCHLEAR NUCLEUS</td>
<td>37</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>37</td>
</tr>
<tr>
<td>METHODS</td>
<td>39</td>
</tr>
<tr>
<td>RESULTS</td>
<td>40</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>48</td>
</tr>
<tr>
<td>CHAPTER 4: GENERAL DISCUSSION AND FUTURE WORK</td>
<td>51</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>55</td>
</tr>
</tbody>
</table>
## Glossary of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABI</td>
<td>Auditory brainstem implant</td>
</tr>
<tr>
<td>ABR</td>
<td>Auditory brainstem response</td>
</tr>
<tr>
<td>ACtx</td>
<td>Primary auditory cortex</td>
</tr>
<tr>
<td>AVCN</td>
<td>Antero-ventral cochlear nucleus</td>
</tr>
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<td>ChR2</td>
<td>Channelrhodopsin-2</td>
</tr>
<tr>
<td>CF</td>
<td>Characteristic frequency</td>
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<tr>
<td>CI</td>
<td>Cochlear implant</td>
</tr>
<tr>
<td>CN</td>
<td>Cochlear nucleus</td>
</tr>
<tr>
<td>DCN</td>
<td>Dorsal cochlear nucleus</td>
</tr>
<tr>
<td>IC</td>
<td>Inferior colliculus</td>
</tr>
<tr>
<td>ICC</td>
<td>Central nucleus of the inferior colliculus</td>
</tr>
<tr>
<td>i.m.</td>
<td>Intra-muscular</td>
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<td>i.p.</td>
<td>Intra-peritoneal</td>
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<tr>
<td>INS</td>
<td>Infrared neural stimulation</td>
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<td>IR</td>
<td>Infrared</td>
</tr>
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<td>NF2</td>
<td>Neurofibromatosis type 2</td>
</tr>
<tr>
<td>PSTH</td>
<td>Peristimulus time histogram</td>
</tr>
<tr>
<td>PVCN</td>
<td>Postero-ventral cochlear nucleus</td>
</tr>
<tr>
<td>RIF</td>
<td>Rate/intensity function</td>
</tr>
<tr>
<td>SI</td>
<td>Synchronization index</td>
</tr>
<tr>
<td>SIF</td>
<td>Synchronization index/intensity function</td>
</tr>
<tr>
<td>SOC</td>
<td>Superior olivary complex</td>
</tr>
<tr>
<td>VCN</td>
<td>Ventral cochlear nucleus</td>
</tr>
</tbody>
</table>
Chapter 1:

Introduction

This scholarly project is concerned with the issue of providing therapeutic options to patients with hearing loss. In particular, one of the missions of our laboratory is to advance treatment of hearing loss for patients eligible for an Auditory Brainstem Implant (ABI) – a neuroprosthesis that directly stimulates the auditory brainstem to create sound perception. In this chapter, we will introduce and define concepts that will be useful throughout this report. In particular, we will give a rationale for the ABI, and explain the difficulties encountered with current technology. We will also describe the basic anatomy and physiology of the auditory system, with a focus on the brainstem nuclei we studied in this project – namely the cochlear nucleus (CN), which we stimulate by electrical or optogenetic means, and the inferior colliculus (IC), where we record responses. In Chapter 2, we present the results of a study on electrical stimulation of the CN, while Chapter 3 demonstrates the feasibility of CN optogenetic stimulation. Finally, Chapter 4 is a general discussion of our project and suggests directions for future work.

According to the National Institute of Health, as many as 36 million Americans (17% of the total US population) are affected by some degree of hearing loss. One in eight Americans over the age of 12 has a bilateral hearing loss (Lin et al., 2011). For some patients who have a significant hearing loss, middle ear surgery, amplifying hearing aids or cochlear implants provide benefit (Bittencourt et al., 2012). A subset of patients do not experience significant improvements from these therapeutic interventions, due to a variety of reasons. One reason is a damaged auditory nerve (AN) due to the growth or removal of a vestibular schwannoma, a benign tumor that causes deafness in patients with Neurofibromatosis Type 2 (NF2). This genetic condition has an incidence of 1:25,000 (Evans et al., 2005), and is usually associated with bilateral schwannomas (Evans, 2009). Other conditions associated with hearing loss that cannot be helped by traditional hearing aids and cochlear implants are a damaged AN due to a temporal bone fracture, ossification of the cochlea as a consequence of bacterial meningitis, congenital absence of the AN (aplasia), or severe cochlear hypoplasia (or aplasia). These patients can potentially benefit from an Auditory Brainstem Implant (or ABI), a device designed to electrically stimulate the Cochlear Nucleus (CN) and second order neurons of the auditory pathways with a surface electrode array. The CN (Fig. 1.1) is the target of the AN and the most peripheral nucleus of the central auditory system (e.g. Ehret and Romand, 1996). By directly stimulating the CN, ABIs bypass the damaged auditory nerve or the ossified/malformed cochlea. In the US, ABIs are approved by the FDA for use in NF2 patients ages 12 and above. In Europe, emerging clinical data suggests that deaf patients who
are not cochlear implant candidates and who do not have NF2 have better outcomes than the average NF2 ABI user (Colletti et al., 2005; 2012). An FDA investigational device exemption clinical trial is underway at Harvard Medical School and the Massachusetts Eye and Ear Infirmary to study the efficacy of the ABI in children and adults who do not have NF2.

The CN is a complex nucleus that has been extensively studied in a variety of animal models. It is anatomically subdivided into 2 main nuclei (e.g. Ehret and Romand, 1996): the ventral cochlear nucleus (VCN) and the dorsal cochlear nucleus (DCN). These subdivisions are characterized by specific cell types and anatomic projections, and are thought to carry out different functions (Oertel, 1991). The VCN is further divided (Fig. 1.2) into the antero-ventral cochlear nucleus (AVCN) and the postero-ventral cochlear nucleus (PVCN). Among the cell types encountered in the AVCN, the bushy cells project to the

**Figure 1.1:** Basic anatomy of the peripheral auditory system and location of the cochlear nucleus in the brainstem
superior olivary complex (SOC) and are thought to be key in the processing of temporal and spatial information. The stellate cells are found in the PVCN, have a characteristic “chopper” response, and send various projections to the CN, the periolivary nuclei of the SOC, and downstream to the inferior colliculus (IC). Octopus cells are another cell type found in the VCN. They have a characteristic “onset” response and likely play a role in processing of temporal information. The DCN contains other cell types and, contrary to the VCN, is organized in layers. The three main cell types of the DCN are the fusiform, giant, and tuberculoventral cells. The fusiform cells project to the IC and may play a role in the processing of spatial information in the vertical plane.

A common feature of auditory neurons throughout the peripheral and central auditory system is

Figure 1.2: Tonotopic organization of the CN in cat (AVCN: antero-ventral CN; PVCN: postero-ventral CN; DCN: dorsal CN). From Ryugo and Parks, 2003.
that they respond preferentially to a specific sound frequency, or characteristic frequency (CF). Neurons of similar CF are grouped together, resulting in spatial gradients of frequency representation – or tonotopy – throughout an auditory nucleus. Figure 1.2 shows the basic tonotopic organization of the cat CN (Ryugo and Parks, 2003). As shown in this figure, tonotopy is inherited from the auditory nerve (AN), and is reproduced in each subdivision of the CN described above (AVCN, PVCN, DCN).

Auditory neuroprostheses try and take advantage of the tonotopic organization of the auditory system. For example, cochlear implants are composed of a linear electrode array coiled inside the cochlea in proximity to the auditory nerve, and each electrode is programmed to provide sound frequency information near the CFs of nearby auditory nerve fibers. In the CN, ABIs also try to deliver frequency-specific information to different neuronal populations. To that effect, a microphone and electronic processor (Fig. 1.3A) externally worn near the ear encode sound into a number of frequency channels. This information is transmitted via an external transmitter coil to a receiver-stimulator unit connected to an array of electrodes (Fig. 1.3B). The electrode array is placed on the surface of the CN by a neuro-otologist or neurosurgeon. A close up of an example array is shown in Figure 1.3C. Each electrode of the array is used to deliver electrical pulses to the CN surface. The frequency channels encoded by the processor are mapped to the electrode array to try and deliver frequency-specific information to groups of neurons located in the vicinity of each electrode contact.

Contrary to cochlear implant users who often show dramatic improvements in their ability to
understand speech, ABI users typically experience little benefit from current ABI technology, except for basic sound awareness and help with lip-reading (Otto et al., 1998; Colletti et al., 2012). Part of the problem may be that electrical stimulation leads to a wide spread of excitation, thereby decreasing the ability to selectively activate small groups of neurons. As frequency resolution is important for speech intelligibility (e.g. French and Steinberg, 1947; Hill et al., 1968; Dubno and Dorman, 1987; Dubno and Schaefer, 1995), selective stimulation of small groups of neurons with specific sound frequencies is crucial to providing clear speech information to ABI patients. However, the wide spread of excitation typically achieved with electrical stimulation may preclude the selective activation of auditory neurons, thereby blurring the speech information. Moreover, many ABI patients experience side effects such as dizziness, facial twitching, or pain, due to electrical stimulation spreading to nearby non-auditory neural structures, such as the facial nerve (Shannon et al., 1993).

Previous studies have shown in animal models that the spatial selectivity of electrical stimulation of the surface of the CN is usually poor (McCreery and Han, 2010), consistent with the difficulty in understanding speech and the side effects experienced by ABI users. Yet, a detailed description of the effects of electrode placement and stimulation pulse rate is lacking in the literature. In Chapter 2, we quantify the spread of excitation elicited by electrical stimulation of CN auditory neurons in a rat model, and report effects of electrode placement and stimulation pulse rate on spatial selectivity and stimulation threshold, in an effort to improve our understanding of electrical ABIs and their limitations. In addition, while current ABIs are made of electrodes embedded in a rigid silicone paddle that does not conform to the convex surface of the human CN, a flexible array may enable better contact and sharper spatial selectivity. Chapter 2 will also present data demonstrating the feasibility of CN electrical stimulation with a new generation flexible electrode microarray (Lacour et al., 2010), designed and manufactured by our collaborating team at Ecole Polytechnique Fédérale de Lausanne (EPFL) in Switzerland.

Instead of electrical stimulation, an optogenetic approach is promising, as light may be more easily focused than electrical current. Optogenetic control of neurons has been demonstrated in a number of systems (e.g. Han et al., 2009), and channelrhodopsin-2 (ChR2), a blue-light responsive ion channel, has successfully been expressed in the CN (Acker et al., 2011; Shimano et al., 2013). Although local brainstem responses have been reported (Shimano et al., 2013), activation of the next stages of the central auditory pathways, such as the inferior colliculi (IC) and the primary auditory cortex (ACtx) has seldom been described previously. In Chapter 3, we report successful activation of IC and ACtx in response to optogenetic stimulation of the CN in a mouse model injected with AAV virus modified to express ChR2. Assuming that narrower regions of the CN can be activated by focusing the light, this work has the potential to dramatically improve the quality of life of ABI users by improving their speech understanding.
ability, while reducing side effects of ABI stimulation. **Our goal is to lay the groundwork for a new generation optogenetic ABI.**

Our approach, for both the electrical and the optogenetic studies, is to measure the response to CN stimulation mostly in one downstream nucleus: the IC (Fig. 1.4A). The IC was chosen because it plays a central role in the auditory system, as an obligatory synapse for virtually all ascending information (Winer and Schreiner, 2005). The main ascending inputs to the IC include projections from the ventral and dorsal CN (mostly contralateral) as well as the medial, lateral, and periolivary nuclei of the SOC, and from the nuclei of the lateral lemniscus (Adams, 1979). The main target of IC neurons is the medial geniculate
body of the thalamus (Wenstrup and Grose, 1995), but there are also numerous descending projections from the IC to various subcollicular nuclei, including the ipsilateral and contralateral DCN (Vater and Feng, 1990). Classic main subdivisions of the IC based on anatomic and physiologic properties of their neurons are the central nucleus (ICC), the dorsal cortex, and the external cortex. The ICC is remarkable in that it is composed of parallel tonotopic laminae (Fig. 1.4B) with CF increasing dorso-laterally to ventro-medially (e.g. Merzenich and Reid, 1974; Winer and Schreiner, 2005).

We take advantage of this laminar tonotopic organization of the IC to quantify the spread of excitation of the CN in our experiments. For both the electrical experiments in rat and the optogenetic experiments in mice, we simultaneously recorded multiunit activity from the IC (Fig. 1.4A) with a 16-channel recording electrode. Since the auditory system is tonotopically organized, stimulation of a narrow region in the CN results in the excitation of neurons preferentially responding to a similar sound frequency (their CF). In contrast, a wide spread of excitation results in activity across neurons with a broad range of CFs. Since the IC is organized in parallel tonotopic laminae, inserting the recording probe perpendicular to the tonotopic axis places each recording contact in different frequency regions (Fig. 1.4B). A narrower spread of excitation in the CN therefore translates in IC activity in a small number of nearby electrode contacts, while broad excitation in the CN results in a greater number of electrode channels displaying IC activity (Fig. 1.4C). Therefore, the spread of excitation in the CN can be assessed through the profile of IC responses across the tonotopic axis.

Overall, this project advances the field by demonstrating a possible new approach to brainstem stimulation that may result in better outcomes for patients. In addition to improving ABI technology and potentially having an impact on the quality of life of ABI patients, our findings improve the basic understanding of electrical and optical stimulation of neurons, and could be extended to other nuclei in the auditory system, as well as other sensory and motor systems for which neural implantation is a potential therapeutic route.
Chapter 2:

Electrical stimulation of the cochlear nucleus

Abstract:
Auditory brainstem implants (ABIs) help provide some hearing to patients with non-functional auditory nerves or ossified cochleae. Yet, performance of ABI users in speech reception tasks is usually worse than for cochlear implant users. ABIs bypass the cochlea and auditory nerve and electrically stimulate the surface of the cochlear nuclear complex. Two of the possible reasons for their limited performance are: (1) the relative imprecision of device placement, making it difficult to specifically target frequency regions important for speech understanding, and (2) the poor spatial selectivity of electrical stimulation, which limits frequency resolution. In this chapter, we evaluate the effects of electrode placement and pulse rate on spatial selectivity and stimulation threshold in the hope of improving ABI performance. The surface of the dorsal cochlear nucleus (DCN) of anesthetized rats was stimulated with either commercially available parylene-insulated platinum-iridium twisted electrodes or with novel, flexible micro-electrode arrays integrated into a polyimide polymer pad. Multiunit activity was recorded with a 16-micro-electrode array in the inferior colliculus (IC). Consistent with the tonotopic organization of the DCN and IC, we found that DCN surface stimulation laterally (low frequencies) to medially (high frequencies) led to lower threshold activation dorsally to ventrally in the IC. Spatial selectivity of electrical stimulation, although somewhat narrower in the rostral-most or caudal-most portions of the DCN, was usually poor. Lower stimulation thresholds were found on the medial side of the DCN. Increasing stimulation pulse rate improved activation thresholds at the expense of temporal precision. Overall, our results point to the need for new stimulation strategies and devices to improve frequency representation in the auditory system of ABI users.

Student contribution:
Aside from flexible electrode microarray design and production by our collaborating team at EPFL, all other work, including animal anesthesia and surgical preparation, stimulus and experimental design, data collection, development of data analysis techniques, data analysis, interpretation, and presentation, was done by the student.

Presentation of findings:
This work was presented in poster form at the Association for Research in Otolaryngology Midwinter Meeting in Baltimore in February 2013 (Slama et al., 2013a), at the 73rd Soma Weiss Student Research Day at Harvard Medical School (Slama et al., 2013b), and some of the data was included in an article published in Hearing Research (Verma et al., 2014).

Introduction
Auditory brainstem implants (ABIs) help provide some hearing to patients with non-functional auditory nerves (from tumors, surgery, or trauma), ossified cochleae, or severe inner-ear dysplasia. In particular, patients with Neurofibromatosis Type 2 (NF2), a devastating genetic syndrome associated with
the growth of multiple brain and spinal cord tumors, have a high risk of developing bilateral vestibular schwannomas. Growth and/or removal of these tumors typically lead to deafness that cannot be helped with cochlear implants.

ABIs are comprised of an externally worn speech processor and a surgically implanted receiver-stimulator with a surface electrode array that bypasses the cochlea and auditory nerve, and electrically stimulates the surface of the cochlear nuclear complex (CN). Contrary to cochlear implant (CI) users, the majority of NF2 ABI users do not achieve open set word recognition (Otto et al., 1998; Colletti et al., 2012). Two of the possible reasons for their limited performance are: (1) the relative imprecision of device placement, making it difficult to specifically target regions important for speech understanding, and (2) the poor spatial selectivity of electrical stimulation, which limits frequency resolution. In addition, ABI users often experience side effects from electrical stimulation of non-auditory pathways, resulting in facial nerve symptoms, dizziness, throat and tongue sensations and limb pain (Shannon et al., 1993).

Previous studies have investigated electrical stimulation of the CN with penetrating microelectrode arrays (Semple and Aitkin, 1980; McCreery et al., 1992; 1998; 2000; McCreery and Han, 2010; Hoa et al., 2008; Shivdasani et al., 2008; 2010), focusing mostly on the ventral CN. However, penetrating ABIs are not approved by the FDA, and the current surgical approach only permits surface stimulation of the dorsal CN. Only a few studies have shown in animal models that the spatial selectivity of CN surface electrical stimulation is usually poor (McCreery and Han, 2010), consistent with the difficulty in understanding speech and the side effects experienced by ABI users. Yet, a detailed description of the effects of electrode placement and stimulation pulse rate is lacking in the literature. One of our goals is to evaluate the effects of electrode placement and pulse rate on spatial selectivity and stimulation threshold in an animal model, in the hope of improving our understanding of electrical ABIs.

Another goal of our research is to use new materials to develop a flexible micro-electrode array for CN surface stimulation (Lacour et al., 2010): While current ABIs are made of electrodes embedded in a rigid silicone paddle that does not conform to the curved surface of the human CN, a flexible array may enable better contact and sharper spatial selectivity. We demonstrate here the feasibility of CN stimulation with a flexible array integrated into a polyimide polymer pad. This project may lay the groundwork for the development of new generation flexible ABI devices.

**Methods**

Anesthesia and surgery

All experimental procedures were performed in accordance with the Animal Care and Use Committee of the Massachusetts Eye and Ear Infirmary. Male Sprague-Dawley rats were deeply anesthetized with ketamine (80 mg/kg i.p.) and xylazine (8 mg/kg i.p.). Level of anesthesia was
monitored by periodically checking toe-pinich reflex, and boosters of ketamine (40 mg/kg i.m.) and xylazine (4 mg/kg i.m.) were administered as needed. After induction of anesthesia, atropine (0.4 mg/kg i.m.) was given to minimize respiratory secretions, and dexamethasone (0.8 mg/kg) was given to minimize brain swelling. A tracheal cannula was then placed to facilitate breathing and the animal was positioned over a warming blanket in a stereotactic apparatus (David Kopf Instruments, Tujunga, CA) with its head stabilized by bars positioned near the ear canals.

A midline skin incision was made over the skull. Soft tissue and neck muscles were dissected away, and a left posterior suboccipital craniotomy was created with a hand drill to expose the dura mater overlying the cerebellum. The dura was incised and a portion of cerebellum was aspirated to expose the fourth ventricle and the surface of the left dorsal cochlear nucleus. The exposed brain tissue was covered with saline and gel foam while performing a second craniotomy superiorly on the contralateral (right) side to provide access to the inferior colliculus (IC) for multiunit recordings.

In animals for which electrical and infra-red co-stimulation of the cochlear nucleus (CN) was studied, unilateral deafening was performed to eliminate responses the infra-red optophonic artifact (Verma et al., 2014), by transecting the auditory nerve as it enters the brainstem.

Flexible stimulating microelectrode array

One of the aims of this study was to test in vivo a novel, flexible stimulating micro-electrode array integrated into a polyimide polymer pad (Lacour et al., 2005; 2010; Graz et al., 2011) designed and built by our collaborating team at Ecole Polytechnique Fédérale de Lausanne (EPFL). The microarrays (Fig. 2.1 A) were manufactured on flexible, thin polymer foils (polyimide). Polyimide is a flexible and long-term biocompatible substrate. The use of a flexible polymer base allowed us to more easily position the array and improve the contact to the curved CN (Fig. 2.1 B). The microelectrodes were made of platinum, which is biocompatible and has good charge injection properties. Because of the electrodes’ small dimension (200 µm), the impedance was too high for neural stimulation, and was decreased to an appropriate 10-20 kΩ by coating the electrode sites with Platinum Black.

Electrical stimulation of the CN

The exposed surface of the CN was electrically stimulated with either the novel microelectrode arrays described above, or using a pair of twisted stainless steel wires insulated except at the tip (“twisted” electrodes, 200 µm diameter; impedance 100-500 kΩ). To study the effect of stimulation location on IC responses in a reproducible way across animals, we used a grid system to map out the surface of the CN into 12 locations (Fig. 2.7A). The twisted electrodes were small enough to enable contact in one of these 12 locations at a time.
For both types of electrodes, current pulses were generated through a stimulus isolator (Model 2200, A-M Systems, Carlsborg, WA). Stimuli were biphasic, bipolar current pulse trains with pulse width 100 μs (for each phase) and amplitudes ranging from 0–0.4 mA. When studying the effect of stimulation location, pulse trains were presented continuously at a rate of 23 Hz. In experiments investigating the effect of stimulation pulse rate, pulse trains were 400 ms long, followed by a 600 ms OFF period with no stimulation (Fig. 2.2 A), and repeated 6 times. In these experiments, stimulation pulse rates ranged from 11 to 513 Hz.

In a few cases, we explored whether stimulating the surface of the CN with infrared (IR) light modulated the effect of electrical stimulation. As detailed in Verma et al. (2014), IR pulses with a wavelength of 1849 nm were generated using a diode laser (Capella R-1850, Lockheed-Martin Aculight Corp., Bothell, WA) and delivered via a 400 μm optical fiber. The optical fiber was positioned on the surface of the CN next to the stimulating electrode. The stimulation paradigm consisted of a 2 ms IR pulse immediately followed by a 200 μs biphasic electrical pulse. The rate of stimulation was 23 Hz.

**ABR measurements**

Auditory Brainstem Responses (ABRs) were measured before and after surgery in response to acoustic clicks delivered at the tympanic membranes (23 Hz repetition rate, 0.1 ms click duration) at levels ranging from 0 – 80 dB SPL. 3 needle electrodes were placed subcutaneously to record the evoked

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**Figure 2.1:** A. Schematic of the novel flexible microelectrode array manufactured by our collaborating team at EPFL. B. One of these stimulating microelectrode arrays on the surface of a rat CN.
potentials (vertex, behind left ear, and ground electrode on the back). ABR signals were bandpass filtered, amplified (Ithaco Model 1201, DL Instruments, Ithaca NY) and averaged (512 repetitions). We proceeded with our experiments only in animals for which ABR thresholds did not change by more than 20 dB SPL after surgery was completed. ABRs in response to electric stimulation (eABRs) were measured in a similar fashion in response to biphasic, bipolar pulse trains.

When an animal was deafened by transecting the AN for the co-stimulation experiments, ABRs in response to acoustic clicks were measured to verify deafening.

IC recordings

Experiments were conducted in a sound-attenuating chamber. A closed-field sound system was placed in the left ear canal. A 16-channel linear electrode array (A1x16-5 mm-150-177, Neuronexus, Ann Arbor, MI), was descended with a micromanipulator (David Kopf Instruments Tujunga, CA) through the contralateral superior craniotomy and inserted in the IC. Placement in the IC was confirmed by recording neural responses to tone pips (20 ms duration, 1 - 45 kHz in 2 steps per octave) and verifying sharp tuning and tonotopic progression along the electrode array consistent with the dorsal to ventral tonotopic organization of the IC (see Fig 2.4).

IC recordings were performed simultaneously to electrical stimulation of the CN. As explained in Chapter 1 in details, the tonotopic organization of the IC is used to quantify the spread of excitation elicited by electrical stimulation: Stimulation of a narrow region in the CN results in the excitation of neurons preferentially responding to a similar sound frequency (their characteristic frequency or CF), which translates into activity in a small number of electrodes of the recording microelectrode array inserted perpendicular to the tonotopic laminae of the IC (see Fig. 1.4).

Data analysis

All data analysis was performed with custom-made programs in Matlab software.

Spike detection: Voltage waveforms of multiunit activity were measured with the 16-channel recording electrodes, amplified, and recorded to disk. To remove slow fluctuations in the signal due to movement or other slow artifacts, the mean waveform across the 16 electrode channels was computed and subtracted from the individual channel waveforms. Additional electrical and physiological noise was further removed by filtering the resulting waveforms between 500 and 3000 Hz with a 5-point Butterworth filter. Spikes were defined as waveform segment that had values greater than 4 times (a factor determined empirically) the background noise level. For the location experiments, background noise was defined as the median value of the filtered waveform in between pulses of the continuous 23 Hz pulse train, starting 15 ms after pulse onset. For the pulse rate experiments, background noise was
defined as the median value of the filtered waveform during the 600 ms period between pulse trains when stimulation was OFF. After detecting the spikes as explained above, neural responses to electrical stimulation were quantified by the average firing rate and the synchronization index.

**Average firing rate response:** Average firing rate in response to electrical stimulation was computed by dividing the number of spikes detected during an analysis window by the duration of the analysis window, averaged over pulse train presentations. For the location experiments with the continuous pulse train, the analysis window was [3 – 15 ms] following pulse onset. For the pulse rate experiments, the analysis window was the 400 ms window during which the pulse train was presented, truncated by 1 ms intervals corresponding to the electrical stimulus artifact (a large voltage peak of less than 1 ms created by propagation of the electrical stimulus to the recording electrode, rather than electrical activity created by neurons in the vicinity of the recording electrode). For each of the 16 electrodes of the recording array, firing rate was plotted as a function of stimulating current intensity and the resulting rate/intensity function (RIF) was fitted with a sigmoid curve.

**Activation threshold and bandwidth:** Spontaneous firing rate was computed for each electrode from the OFF portion of the response (corresponding to the interval used to compute the background noise as defined above). Evoked firing rates were compared between the analysis window and the OFF portion across various pulse rates and pulse amplitudes with a paired t-test. For each electrode number, the logarithm of the p-values was plotted as a function of current intensity and fitted with a sigmoid curve. Activation threshold was determined as the current intensity at which the fitted log-p-value/intensity function became smaller than 0.01. Spread of excitation in the IC was determined by the activation bandwidth, defined as the number of electrodes for which activation threshold was crossed.

**Temporal response:** To investigate temporal properties of responses to electrical stimulation, we computed the Synchronization Index (SI; Goldberg and Brown, 1969). The SI is a measure of the precision of the response in the time domain for periodic stimuli such as a pulse train. It varies between 0 and 1: Spikes occurring at completely random phases of the stimulus pulse train lead to a synchronization index of 0, while spikes occurring reliably at the same phase of the stimulus pulse train lead to an index of 1. For each electrode, SI was plotted as a function of stimulating current intensity and fitted with a sigmoid curve (synchronization index/intensity function or SIF). A Rayleigh test of uniformity was used to assess the significance of the computed SI (Mardia and Jupp, 1999).

**Synchronization threshold and bandwidth:** Similar to our determination of activation threshold, the logarithm of the p-values obtained with the Rayleigh tests was plotted against current intensity and fitted with a sigmoid curve. Synchronization threshold was determined as the light intensity at which the fitted log-p-value/intensity function crossed 0.01. Synchronization bandwidth was defined as the number of electrodes that crossed synchronization threshold.
Results
Spatial selectivity of electrical stimulation is poor

We measured multiunit neural activity in the IC in response to electrical stimulation of the CN in 6 rats, using either steel “twisted” electrodes or novel flexible microelectrode arrays made by our collaborating team at EPFL (Methods). Figure 2.2 shows an example response in a rat (ms21) using biphasic electrical pulse trains at 23 Hz delivered via one of the flexible arrays at the surface of the CN. The stimulus is shown in panel A and composed of a 400 ms pulse train followed by a 600 ms OFF period with no stimulation. Below the stimulus is plotted the neural response as a function of stimulus current intensity and repetition number (spike raster) in one of the recording electrodes (#6). At low current levels, spikes can be seen both during the ON and OFF phases of the stimulus, with no appreciable difference between the 2 phases, consistent with spontaneous activity unrelated to stimulation. With increasing current level, there is a clear increase in the number of spikes during the ON phase, within 10 ms of each stimulus pulse. This was replotted in Figure 2.2B as average firing rate in the ON period as a function of stimulation current intensity. Firing rates as a function of intensity was fitted by a sigmoid curve (Methods). The resulting rate/intensity function (RIF) has a threshold near 40 μA and saturates around 0.25 mA.

Figure 2.2C shows average firing rate as a function of current intensity and IC electrode number as a color map. In this plot, the baseline firing rate of each electrode was subtracted to facilitate comparison across electrodes. High electrode numbers were located dorsally in the IC and correspond to low CF laminae, while low electrode numbers correspond to higher CF. Activation is seen in all electrodes. In this example, higher firing rates are observed at high CF. When computing activation thresholds across electrodes, we can see that the activation bandwidth was very broad (Fig. 2.2D) with a significant increase in firing rate in all recording electrodes. Activation thresholds were very similar across electrodes (ranging from about 25 to 75 μA) in spite of large differences in saturation rates (higher rates at high frequencies in Fig. 2.2C). This broad activation of the entire tonotopic axis in the IC while stimulating only one region of the CN is consistent with a broad spread of excitation of electrical stimulation.

Comparison between electrical and acoustic stimulation

We showed in the previous section that the IC was broadly activated when stimulating the surface of the CN with the flexible microelectrode array in an example. One of the questions raised with this new neuroprosthesis is its ability to generate even higher order responses that are recognizable and useful from a clinical standpoint. ABRs have been used for years both clinically and in research settings to study or elucidate hearing status in individuals. The various peaks of the evoked waveforms are thought to be
related to the sequential activation of the different auditory nuclei. We measured ABRs in response to electrical stimulation in some of our experiments. Figure 2.3 compares the acoustically evoked and

Figure 2.2: Example rat (ms21) showing broad activation across the IC in response to a 23Hz biphasic electrical pulse train delivered at the surface of the CN.  A. Dot raster from electrode #6.  B. Rate-intensity function (RIF) showing average firing rate as a function of current intensity from electrode #6.  C. Average firing rate as a function of current intensity and IC recording electrode.  D. Activation thresholds as a function of IC recording electrode.
electrically evoked ABR in the same example animal as Figure 2.2. In response to acoustic clicks (left panel), a classic acoustic ABR is recorded, with a threshold near 35 dB SPL. In the same animal and in response to electrical stimulation of the CN at the same pulse rate (23 Hz), the ABR (right panel) shows a multi-peak response within the first 7 ms after stimulus onset, with a threshold near 50 μA. The ABR waveform pattern was overall different between modes of stimulation, in particular in the first 2-3 ms. This difference in patterns especially near the onset is likely a reflection of the different stages at which the auditory system was stimulated, resulting in shorter latency in the electrical case, with possibly antidromic propagation back to the auditory nerve in addition to forward propagation through the auditory pathway. Of note, the sharp biphasic onset of the electrical ABR waveform is an electrical stimulus artifact. These ABR recordings show that the novel EPFL microelectrode arrays are able to generate ABRs at low thresholds.

**Figure 2.3:** Auditory Brainstem Responses measured in response to acoustic clicks (left) and electrical pulses (right) in an example (rat ms21). Evoked potentials were clearly present in the first 7 ms after stimulus onset.
In the same animal, IC recordings of acoustic tones delivered at the ear canal show the expected tonotopic progression: In Figure 2.4, there is clear progression of the area of maximum response from dorsally to ventrally placed electrodes as tone frequency varies from low (1 kHz) to high frequency (45 kHz). The narrow bandwidth of activity recorded in response to acoustic tones is in stark contrast to the broad activity described in the same animal in response to electrical stimulation as shown in Figure 2.2. The sharp tuning and tonotopic progression measured in response to acoustic stimulation with tones is also consistent with the recording electrode being placed in the central nucleus of the IC.

Figure 2.4: IC recording in the same animal as figure 2.2 and 2.3 (rat ms21) in response to acoustic tones delivered at the eardrum. Contrary to electrical CN stimulation (see Fig. 2.2), responses to tones exhibit sharp tuning.

In the same animal, IC recordings of acoustic tones delivered at the ear canal show the expected tonotopic progression: In Figure 2.4, there is clear progression of the area of maximum response from dorsally to ventrally placed electrodes as tone frequency varies from low (1 kHz) to high frequency (45 kHz). The narrow bandwidth of activity recorded in response to acoustic tones is in stark contrast to the broad activity described in the same animal in response to electrical stimulation as shown in Figure 2.2. The sharp tuning and tonotopic progression measured in response to acoustic stimulation with tones is also consistent with the recording electrode being placed in the central nucleus of the IC.

Strong synchronization to electrical pulses

We showed in previous sections that electrical stimulation of the CN using the novel flexible microelectrode array lead to significant activation of the IC with low thresholds throughout the tonotopic axis, consistent with broad spread of excitation of electrical stimulation. We also showed that this activation led to detectable evoked potentials that appropriately differed from acoustically evoked ABRs.
Figure 2.5: Same example rat (ms21) showing broad synchronization across the IC in response to a 23Hz biphasic electrical pulse train delivered at the surface of the CN. 

A. Peristimulus Time Histogram from IC electrode #6. 
B. Synchronization index as a function of current intensity from electrode #6. 
C. Synchronization index as a function of current intensity and IC recording electrode. 
D. Synchronization thresholds as a function of IC recording electrode
Another way in which the response to electrical stimulation can be quantified is by studying its temporal properties. In the same example case as previous figures (rat ms21), Figure 2.5A shows a Peristimulus Time Histogram (PSTH) in response to the 23 Hz biphasic pulse train as a function of current intensity. Phase-locking to the stimulation pulse rate is seen at most stimulation currents, except at the lowest where spiking was random. When quantifying phase-locking with the synchronization index (SI), and plotting SI as a function of stimulation current, the resulting synchronization index/intensity function (SIF, see example electrode #6 in Fig. 2.5B) rises sharply before plateauing around 0.15 mA. The saturation of the SIF occurs at lower currents than that of the RIF for the same data. SI in the plateau is close to 0.8, which shows sharp temporal response of the auditory system in response to electrical pulses. The synchronization threshold in this example is comparable to the activation threshold, around 40 μA.

When plotting SI as a function of current intensity across all electrodes (Figure 2.5C), a pattern similar to that of electrode #6 is present throughout the tonotopic axis: Low synchronization threshold, early saturation of SI, and overall sharp temporal response with high SI throughout. The synchronization thresholds were similar across electrodes (Figure 2.5D), ranging from about 20-45 μA. The synchronization thresholds in this example were overall lower than activation thresholds, which may reflect the known tendency of auditory neurons to organize their spontaneous activity in phase with periodic stimuli at low stimulus intensity before raising their firing rates, at stages as early as the auditory nerve (Johnson, 1980).

Effects of pulse rate

While the previous sections focused on responses at one pulse rate (23 Hz) in an example case, we study here the effect of stimulation pulse rate on various metrics derived from the IC responses. The same example (ms21) is used to illustrate representative effects of pulse rate in Figure 2.6. We varied pulse rate from 11 Hz to 253 Hz with approximately 1 to 2 steps per octave. At low stimulation pulse rates (Figure 2.6A, top left panels), average firing rate was overall low and similar across recording electrodes (or CF locations). Firing rates steadily increased with increasing stimulation pulse rates, with more activity recorded in electrodes located in high CF regions in this example.

The dependence of average firing rate on stimulation pulse rates can be better appreciated in individual electrodes (see electrode #4 in Figure 2.6B, top graph). Individual RIFs approximated sigmoid shapes, with plateau firing rate increasing monotonically with stimulation pulse rate. This dependence of firing rate on pulse rate is expected, as the number of stimulation pulses per unit time increases with increasing pulse rate. Of note, the activation thresholds also depended on pulse rate, with lower thresholds found at higher pulse rates. This is also expected given the larger stimulation energy delivered at higher pulse rates, due to the greater number of current pulses per unit time.

25
We also studied the effect of pulse rate on the temporal response in the same example case. Figure 2.6A (bottom panels) shows strong synchronization to the lowest stimulation pulse rates. However, the temporal response seems to break down with increasing pulse rates, suggesting that the increased rate response comes at the expense of temporal precision. SI at the highest pulse rates was not sufficiently synchronized due to electrical stimulus artifact at these high frequencies.

Figure 2.6: Effects of stimulation pulse rate on IC responses. **A.** Activation and synchronization maps in an example rat (ms21). **B.** Average firing rate (top) and synchronization index (bottom) as a function of current intensity and pulse rate in electrode #4. **C.** Population summaries across animals and IC electrodes.
shown because our techniques could not provide a reliable estimate of SI at high pulse rates due to the stimulation artifact. As explained in the Methods, SI is a measure of how synchronized the spike train is to the periodic stimulus phase. The strong stimulus artifact created by propagation of current from the stimulation site to the recording electrode, unrelated to neuron spiking, is in itself a periodic signal. For our analysis, we removed data in the vicinity of the stimulus artifact so as not to count it as periodic spikes. While this processing does not create a large bias of our SI estimates at low pulse rates, because of the short truncation window compared to the period of the stimulus, this is not the case at high pulse rates, where truncating 1 ms of spikes artificially makes the remaining spikes “look” synchronized, as random as they may be a priori.

When plotting the SIF for an individual electrode (#4) and a range of pulse rates, we see a decrease in the plateau SI for pulse rates above 63 Hz, but very similar SIFs for pulse rates below 43 Hz. There is no clear trend in how synchronization thresholds varied with pulse rates, although the highest pulse rate (123 Hz) clearly resulted in a lower synchronization threshold. As a whole, as observed in a previous section, synchronization thresholds were lower than activation thresholds in the same animal, and SIFs reached their plateau at lower current intensities than RIFs did for the same pulse rate.

We looked at 3 metrics of the IC response across our dataset (maximum response or plateau, threshold, and bandwidth) for both average firing rate and SI, and compared 2 pulse rates (11 Hz and 63 Hz). The results are displayed in Figure 2.6C. For average firing rate response, there was a significant increase in maximum response across all animals and electrodes in the high pulse rate condition compared to low pulse rate (top left panel; mean difference: 263 sp/s; p < 0.001 in a paired t-test). This effect was seen in the individual case described above, and is again consistent with the increased number of stimulation pulses delivered per unit time at higher pulse rates. This difference in stimulation energy can also be invoked to explain the significantly lower activation thresholds (middle left panel, p < 0.001 in a paired t-test) found at high pulse rates (mean threshold: 86 µA) compared to low pulse rates (mean threshold: 147 µA). Across all cases, there was no significant difference in activation bandwidth across pulse rate condition (bottom left panel, p = 0.48 in a paired t-test). Overall, these results confirm the ability of the novel EPFL electrode array to stimulate the CN with low stimulation thresholds, but demonstrate the very broad spatial selectivity of electrical stimulation, as activation bandwidth was close to 16 in all cases, regardless of pulse rate (bottom left panel).

In terms of the temporal response to electrical stimulation, pulse rates had a clear effect as well at the population level. Plateau SI of the SIF was on average higher at low pulse rates than at high pulse rates (top right panel; mean difference in SI: 0.14; p < 0.001 in a paired t-test). There was also a significant effect of pulse rate on synchronization threshold (middle right panel, p < 0.001 in a paired t-test): Thresholds in the high pulse rate condition (mean threshold: 49 µA) were significantly lower than in
the low pulse rate condition (mean threshold: 95 µA). There was no significant difference in synchronization bandwidth across pulse rates (bottom right panel, p = 1 in a paired t-test). Overall, electrical stimulation of the CN with the novel EPFL electrode led to sharp temporal responses in the IC regardless of pulse rate.

Effects of stimulation location

We explored the effects of the location of CN electrical stimulation on IC rate responses. Because the EPFL electrodes we used had a pad with dimensions about half of the exposed CN surface, we were not able to move them around the surface effectively to study the effect of location. Instead, we used the smaller “twisted electrodes” described in the Methods. To compare effect of location across animals, we divided the surface of the exposed CN into 12 distinct locations, similar to what was done in previous studies in our laboratory (Verma et al., 2014). The grid system used to define these locations is shown in Figure 2.7A. It is made of 3 lines named A-C rostrally to caudally, and 4 columns named 1-4 laterally to medially. An example of IC responses to electrical stimulation at 23 Hz in a rat (ms14) across CN locations is shown in Figure 2.7B. There were clear differences in activation threshold, bandwidth, and overall pattern in both dimensions. In this particular example, spatial selectivity was narrower laterally than in the central portions. There was a larger firing rate response in the central and caudal areas than laterally or medially. The most rostral areas were not very responsive except laterally.

Figure 2.7: Effects of stimulation location on IC responses. A. Grid system used to define location across the exposed surface of the CN. B. Example responses in the IC as a function of CN stimulation location (rat ms14). C. Population summary showing lower thresholds medially. Solid line: linear regression.
Across animals, 2-way ANOVA analysis (Table 2.1) suggests that threshold depended on location in the lateral-medial axis (p = 0.0063), but not in the rostro-caudal axis. Across animals, electrodes, and rostro-caudal location (Fig. 2.7C), there was a significant negative correlation between thresholds and lateral-medial location (r = -0.47), suggesting lower thresholds on the medial side. ANOVA analysis across animals also shows preferential activation of more ventral parts of the IC when the medial side of the CN was stimulated (p = 0.0011), consistent with the tonotopic arrangement of these nuclei. Tonotopy was not detected in the rostro-caudal dimension. Selectivity of electrical stimulation, although broad, was somewhat narrower in the rostral-most or caudal-most portions of the DCN (p < 0.001); there was no dependence of bandwidth on location in the lateral-medial axis. Although significant, these effects were overall small and may be influenced by stimulation electrode angle and pressure onto the CN surface.

Effect of co-stimulation with IR

In previous sections, we showed that electrical stimulation of the CN leads to broad activation of the tonotopic axis. Pulse rate and stimulation location had overall small effects on spatial selectivity. Another mode of stimulation investigated in our laboratory is infrared (IR) light. In prior research (Verma et al., 2014), our laboratory established that infrared neural stimulation (INS) of the CN does not produce detectable multiunit responses in the IC, although INS pulses produce a loud optophonic artifact (an acoustic click created by the sudden change of air temperature produced by IR light) whose response can be easily detected in the IC as well as in ABRs. In Verma et al.(2014), although no activation from INS per se could be detected, it is possible that the multiunit recording technique they used was not appropriate to detect small responses, or that INS produces inhibitory responses in the CN. We therefore tested in the current study whether INS can modulate electrical activation of the CN with a bipolar stimulating electrode, as changes in electrical activation may be more easily detected with multiunit recordings. To ascertain that any effects of INS on the responses to electrical stimulation were not due to

<table>
<thead>
<tr>
<th></th>
<th>Threshold</th>
<th>Electrode of maximum</th>
<th>Bandwidth</th>
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<tbody>
<tr>
<td>Rostro-caudal axis (A-C)</td>
<td>p = 0.1497</td>
<td>0.175</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Latero-medial axis (1-4)</td>
<td>p = 0.0063</td>
<td>p = 0.0011</td>
<td>p = 0.172</td>
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Table 2.1: p values of 2-way ANOVA analysis showing dependence of various firing rate metrics on location in the rostro-caudal and lateral-medial axes.
the optophonic artifact, we unilaterally deafened the animal by transecting the auditory nerve on the side of CN exposure. After transection, no ABR or IC responses were detected in response to INS alone with the INS laser placed on the surface of the CN, ensuring that any effect of INS would be due to INS itself rather than the optophonic artifact.

We then positioned a bipolar stimulating electrode next to the optical fiber on the surface of the CN and co-stimulated with the paradigm described in the Methods (2 ms IR pulse followed by 200 µs biphasic electrical pulse, repeated at 23 Hz). To limit biases resulting from changes in baseline IC activity over time, we alternated between stimulation sequences with the INS laser ON, and sequences with the laser OFF. Figure 2.8A shows an example RIF, recorded from 1 electrode from the IC multichannel electrode array. The 4 blue traces show the responses when the laser was OFF, while the 4 red traces show the responses when the laser was ON. There is a change in baseline activity over time as evidenced by the shift of the response curve. However, since ON and OFF conditions were alternated, there is a clear effect of INS stimulation as well. In this example, INS tended to decrease firing rate for a given electrical current, as well as raised activation thresholds by about 50µA.

The mean response curve across electrodes and repetitions is shown in Figure 2.8B for the laser ON and laser OFF conditions. Similarly to the example of Figure 2.8A, firing rate increased monotonically with current levels. Two main effects of INS stimulation were seen on the response curve:
(1) The mean firing rate across electrodes and repetitions decreased from 82 spikes/second for electrical stimulation alone to about 50 spikes/second in co-stimulation. (2) There was a rightward shift of the response curve by about 10-15 µA. Both the vertical and horizontal shifts were statistically significant across electrodes in a paired t test (p < 0.001 for the decrease in mean firing rate, and p = 0.0013 for the rightward threshold shift). Both effects are consistent with INS having inhibitory effects on CN neurons (or activating preferentially inhibitory CN neurons).

**Discussion**

We investigated electrical stimulation of the surface of the CN by simultaneously recording multiunit activity in the IC in anesthetized rats. We found that electrical stimulation of the CN elicited an increase in firing rate with fairly low stimulation thresholds, and that the response activity was synchronized to the pulse rate of the input. Consistent with a broad spread of excitation, the activation bandwidth in the IC was very broad when stimulating only one location of the CN. Spatial selectivity was marginally better in the rostral-most or caudal-most portion of the exposed CN. Increasing pulse rate tended to increase firing rate and decrease synchrony. In one animal, co-stimulation with IR light increased electrical stimulation threshold and deceased maximum firing rate.

**Spatial selectivity of electrical stimulation**

Stimulation with electrical pulses in one location of the CN led to broad activation in the IC, as most, if not all, electrodes were activated, corresponding to multiunit activity detected throughout the entire tonotopic axis (Fig. 2.2C-D). This is consistent with a previous report in the literature that showed broad tuning when using surface electrodes (McCreery and Han, 2010). This poor spatial selectivity may be one factor explaining the difficulty of ABI users to utilize information delivered to the surface of the CN: Frequency information is crucial for speech understanding (e.g. French and Steinberg, 1947; Hill et al., 1968; Dubno and Dorman, 1987; Dubno and Schaefer, 1995), and current ABI technology may not be able to deliver frequency specific information to select populations of neurons with specific CFs. After dividing the acoustic spectrum into a few frequency bands, and mapping these frequency bands onto individual electrodes located over different frequency regions of the DCN, our experiments would suggest that the entire surface of the DCN will be stimulated by each electrode. As a result, all the neurons will receive not only the frequency information that they were supposed to receive, but also the information destined to neurons of other frequency regions. Stimulation is therefore quasi-homogenous across the tonotopic axis, with no frequency resolution.

One could argue that our finding is biased by the fact that we defined the activation bandwidth as the number of electrodes crossing activation threshold – regardless of the current at which threshold is
reached. This definition could be an issue as the likelihood of crossing threshold increases with increasing stimulation current, and therefore for high enough stimulation currents, even a response with sharp tuning at low currents will have a broad activation bandwidth at high currents. However, in our results, it is striking that thresholds were very similar across electrodes, even if there were differences in absolute firing rate across electrodes (see Fig. 2.2C-D). This means that the broad activation bandwidths are not an artifact of our definition, but rather a reflection of the broad spread of electrical current.

More than indiscriminately stimulating neurons of various CFs, current spread is likely not limited to the surface of the DCN. Rather, current may reach deeper regions of the DCN and potentially the ventral CN nuclei (AVCN and PVCN). While stimulating deeper CN neural circuits may be an appealing idea, as the DCN is likely involved in only a subset of auditory functions, the indiscriminate activation of all cell types may pose a challenge to the auditory system rather than provide valuable auditory information. Although AN responses are fairly similar in pattern across AN fibers, and therefore many CN cell subtypes may receive similar inputs, there are also interneurons in the CN and local circuits whose normal physiologic function may depend on receiving very specific patterns of action potentials. In short, current ABI technology may be failing not only in that there is not much frequency resolution, but also because the stimulating schemes are based on CI stimulation, with no differentiation of cell types and normal physiologic responses to imitate.

In an effort to limit current spread during electrical stimulation, the size and distance between the two contacts of the bipolar electrodes should technically be reduced. In our case, this distance was smaller than 500 $\mu$m. One issue at stake is that reducing the electrode size results in an increase in electrode impedance, and therefore higher stimulating currents will be needed to elicit a response. This trade-off needs to be carefully weighed as battery life is a major consideration in neuroprotheses. Further, increasing impedance may lead to a significant decrease in dynamic range, and therefore limit neural encoding in another dimension.

Temporal response to electrical stimulation

An encouraging result of this study is the high synchronization measured in response to pulse trains in the IC. In most cases, pulse trains commonly elicited SIs around ~0.8, close to the maximum of 1 corresponding to perfect synchronization to the pulse train. This is somewhat surprising as we recorded multiunit activity rather than single units. It would be expected that multiunit activity be less well synchronized than single-units, because even if single-unit respond preferentially at one phase of the stimulus, the preferred response phase and overall pattern of the response likely varies greatly across units. There is therefore no guarantee that the resulting multiunit activity keeps a high degree of synchronization. This phenomenon should however be less pronounced for low stimulation pulse rates,
i.e. when the stimulation period is long compared to the span of the neural response. If the neural response is concentrated within 10 ms of the stimulus pulse, as we usually see in our data, the SI will automatically be high for long periods. For example at 5 Hz, the period is 200 ms, and therefore activity is concentrated in less than 5% of the stimulus period. Conversely, for a shorter period (faster pulse rate), the same 10 ms become an increasing portion of the stimulus period. For example at 100 Hz, the stimulus period is also 10 ms, and therefore the SI should break down.

In our data, we do see significant SI at pulse rates as high as 63 Hz, albeit with a significant decrease in SI compared to the 1 Hz condition. It was difficult to explore the ability of the system to synchronize to even faster pulse rates because of the stimulus artifact. As explained in the results, a large stimulus artifact is recorded at each stimulation cycle due to conduction of current from the stimulating electrode to the recording electrode through brain tissue that is not related to action potentials. This artifact biases the SI measure, as it is detected as a spike and artificially introduces synchronicity in the response. Removing the artifact can also bias the SI, because it artificially prevents spikes from occurring near stimulus onset, which also introduces synchronicity. This is less of an issue at low pulse rates, when the duration of the artifact is small compared to the stimulus period, but the bias is significant at high pulse rates, typically above 123 Hz in our dataset.

The ability to synchronize with high SI to pulse rates below 63 Hz is an encouraging result for electrical ABIs. Temporal information is crucial in speech understanding, as demonstrated in numerous studies (e.g. Houtgast et al., 1980; Shannon et al., 1995; 1998). CI and ABI encoding strategies utilize this information, in particular by encoding the amplitude envelopes of auditory signals. Psychophysical studies have shown that low frequencies in the amplitude envelope of speech signals provide adequate cues for speech understanding, even with extremely limited spectral frequency information (Shannon et al., 1995; 1998; Apoux and Bacon, 2004; 2008). Therefore our results support the use of electrical ABIs to transmit useful temporal information, and may explain the ability of some ABI users to achieve some level of speech understanding (Colletti et al., 2009).

Effect of stimulation location

In our dataset, stimulation location did not have much reproducible effects on firing rate or synchrony. Spatial selectivity was marginally better in the rostral-most or caudal-most portion of the exposed CN. These differences may be the result of differences in brain/electrode contact across stimulation locations. As seen on Figure 2.7A, the surface of the CN is curved, and even though the electrode was approached to the surface from a similar angle across location, the center portion allowed a more direct contact than the caudal-most or rostral-most portions. This may be experimental evidence of some issues encountered when using current ABI technology in humans, as current ABI electrode arrays
are typically rigid, and do not conform to the curved surface of the CN, therefore introducing some level of heterogeneity in how electrodes interact with the neural tissue.

Another effect of stimulation location that we detected is the preferential activation of more ventral parts of the IC when the medial side of the CN was stimulated. This is consistent with the tonotopic arrangement of these nuclei, as ventral IC and medial DCN correspond to lower CF (Muniak et al., 2013; Spirou et al., 1993; Merzenich and Reid, 1974; Winer and Schreiner, 2005). However, this effect was only detected when pooling data across all animals, and was not generally obvious in individual animals. This is again an indication that electrical stimulation does not provide adequate frequency resolution to the auditory system.

Feasibility of flexible microelectrode array

One of the goals of this study was to test in vivo the feasibility of using a flexible microarray for electrical stimulation of the CN. The rationale for using a flexible array comes from the idea that the CN is curved and that the spread of electrical current may be greater for rigid arrays that do not conform to this curvature. We did not directly test this hypothesis in this study, but we showed that the novel flexible microarray designed and built by our collaborating team at EPFL was able to elicit activity in the IC at low thresholds and with reliable temporal characteristics. The responses were similar to those elicited by acoustic clicks and the microarrays also generated measurable ABRs that could be used in the operating room to confirm electrode placement.

The microarrays were manufactured on polyimide, a thin, flexible polymer foil that is biocompatible and could be used in chronic experiments and potentially in humans. One limitation of the current design is the need to use Platinum Black to coat the platinum electrodes so that their impedance decreased to a level appropriate for neural stimulation. Platinum Black is not biocompatible, and other materials or techniques will need to be investigated before considering a trial in humans.

Finally, although we did not directly test if having a conformable microarray was of any benefit for frequency selectivity, indications in this study would actually point to the opposite result: In our experiments with the twisted electrodes, there was somewhat better frequency selectivity in the caudal-most and rostral-most regions of the exposed DCN. It is likely that this result was due to poorer contact with the electrode in these curved regions, therefore leading to a smaller surface area of neural-prosthesis interface and less current spread. With that logic, a curved array would lead to more current spread by authorizing all electrodes across the surface to be in maximal contact with the tissue. Only future experiments comparing rigid and conformable electrode arrays will answer this question unequivocally.
Does Infrared Neural Stimulation have an inhibitory effect?

Responses to INS have been reported in only a few papers. Importantly, Verma et al. (2014) showed that responses to INS of the CN measured in the IC disappeared after transecting the AN. This is likely a result of an optophonic artifact of the laser (Teudt et al., 2011): When IR pulses are generated, a sudden local change in air temperature at the output of the optical fiber generates a sound wave that propagates to the ear canal and produces a measurable physiologic response in the auditory system. After deafening the animal by transecting the AN, no responses could be measured in the IC, and no ABR was detected with subcutaneous electrodes, suggesting that INS in itself is not a suitable mode of neurostimulation of the CN.

Verma et al. (2004) used a multiunit recording technique in the IC. While this is appropriate to detect gross changes in neural activity, it is possible that INS does have a small effect that was not detected, or that it has an inhibitory effect on CN cells. To investigate this possibility, we co-stimulated with electrical pulses and INS by placing the stimulating electrode and the optical fiber next to each other on the surface of the DCN. We only have results in one animal, because of the difficulty of keeping a stable preparation after transecting the AN. But we do seem to have a significant effect of INS in this deafened animal, which tended to raise threshold and decrease firing rate in response to electrical stimulation. This would support an inhibitory effect of INS.

A caveat of our result is that we deafened the animal unilaterally, as the contralateral CN was not exposed. Technically, the optophonic artifact produced by the IR laser could travel to the contralateral ear, and the apparent inhibitory effect of INS could be a result of contralateral acoustic stimulation suppressing the cochlea (Guinan, 1996). This is however unlikely for 2 reasons: (1) In Verma et al (2004), responses to the optophonic artifact substantially decreased as the laser was moved more medially, suggesting that bone conduction through the nearby temporal bone when the laser was positioned laterally played a fundamental role. (2) We measured ABRs in response to INS both on the ipsilateral and the contralateral side, and we were not able to detect neither ipsilateral nor contralateral ABRs after unilaterally deafening on the ipsilateral side.

Obviously this inhibitory effect of INS will need to be confirmed in further experiments, but if it is, it opens exciting prospects for ABI technology, as IR pulses could then technically be used to sharpen electrical responses. For example, an opto-electronic array could be designed with IR optodes flanking stimulating electrodes, and therefore sharpen the spatial selectivity (i.e. the frequency resolution) of electrical stimulation by inhibiting areas surrounding an electrode. This design would need to be tested in animals, and translation to a clinical application would likely be arduous as the hardware necessary to generate IR pulses is large, heavy, costly, and energy demanding.
Conclusions

In summary, we showed in rats that electrical stimulation of the CN has poor spatial selectivity but elicits fairly sharp temporal responses, as measured in the IC. We demonstrated that flexible, polyimide based electrode microarrays can provide low stimulation thresholds and elicits measurable evoked potentials. Increasing pulse rate of stimulation lowered activation threshold at the expense of temporal precision, and there was some evidence of stimulation location dependence of the responses, although these effects were small and highly variable. The use of INS to stimulate the CN in conjunction with electrical stimulation suggested an inhibitory effect of INS in one animal.
Chapter 3:

Optogenetic stimulation of the cochlear nucleus

Abstract:
In Chapter 2, we demonstrated that electrical stimulation of the cochlear nucleus (CN) is not very selective, leading to poor frequency resolution in the auditory system, as measured in the inferior colliculus (IC) of rats. This spread of electrical current may play a fundamental role in the typically poor performance of Auditory Brainstem Implant (ABI) users in speech reception tasks. Light may be more easily focused than electrical current, and optogenetics may therefore provide a solution to this resolution problem. In this chapter, we present results showing the feasibility of optogenetic control of the auditory brainstem. The CN of CBA/J mice was transfected with AAV2/8-Channelrhodopsin-2 (ChR2). Following an incubation period of two to four weeks, the CN surface was stimulated with blue light while recording neural activity in the contralateral IC, and occasionally in primary auditory cortex (ACtx). We found a significant increase in IC activity during blue light stimulation in mice with ChR2 expression in the CN. In contrast, no significant increase in neural firing was seen in the IC of mice with failed ChR2 expression in the CN or in non-transfected control mice. Stimulation with high pulse rates (320 Hz) elicited larger firing rates compared to a low pulse rate condition (5 Hz), but did not significantly affect activation thresholds or bandwidths. Synchronization to the stimulus pulse train was degraded at high pulse rates. Overall, these results demonstrate the feasibility of optogenetic control of the auditory brainstem and may lay the groundwork for a new generation opto-electronic ABI.

Student contribution:
Stimulation paradigm, development of customized data analysis techniques, data analysis and interpretation, preparation of figures for publication (Darrow et al., 2014), contributions to manuscript writing, were done by the student. Virus injections, experiments and histology were performed by Dr. Keith Darrow, Maryanna Owoc, and Dr. Judith Kempfle. Other contributors to this study and its publication were Dr. Elliott Kozin, Dr. Kenneth Hancock, Dr. Albert Edge, Dr. Stéphanie Lacour, Dr. Edward Boyden, Dr. Daniel Polley, Dr. Christian Brown, and Dr. Daniel Lee. This chapter is adapted from Darrow et al. (2014) to only reflect the student contribution.

Presentation of findings:
This work was presented in poster form and in a talk at the Association for Research in Otolaryngology Midwinter Meeting in Baltimore in February 2013 (Darrow et al., 2013a; 2013b), in poster form at the 73rd Soma Weiss Student Research Day at Harvard Medical School (Slama et al., 2013b), and recently published (Darrow et al., 2014).

Introduction
As detailed in Chapters 1 and 2, Auditory Brainstem Implants (ABIs) help provide some sense of hearing to patients with damaged auditory nerves or ossified cochleae, by electrically stimulating the surface of the cochlear nucleus (CN). In the US, most ABI users received this type of implant after
removal of a vestibular schwannoma. Contrary to cochlear implant (CI) users, ABI users typically derive limited benefit from their implant in terms of speech understanding ability (Otto et al., 1998; Colletti et al., 2012). One of the possible reasons for this poor performance is the spread of electrical current, which limits spatial selectivity. Because the auditory system is organized tonotopically, poor spatial selectivity translates into reduced frequency resolution. This degradation in frequency information limits one's ability to understand speech (e.g. French and Steinberg, 1947; Hill et al., 1968; Dubno and Dorman, 1987; Dubno and Schaefer, 1995).

In Chapter 2, we showed that electrical stimulation in one location of the CN surface often leads to activation of the entire tonotopic axis, as evidenced by significant increases in activity throughout the entire dorso-ventral axis in the inferior colliculus (IC). This is consistent with the hypothesis that electrical stimulation leads to broad current spread and dramatically reduces frequency resolution. An alternative option would be to use light for neural stimulation, as light may be more easily focused than electrical currents. Infrared neural stimulation (INS) does not in itself provide a valid alternative, as no significant activation of the auditory system could be detected in a study of INS in the CN (Verma et al., 2014). Another approach would be to use optogenetics for neural stimulation of the CN. By delivering light-sensitive ion channels (or opsins) to neural membranes via viral vectors (Boyden et al., 2005) or tissue-specific promoters (Zhao et al., 2011), neurons can be made to depolarize in response to a light stimulus. Optogenetic control of neurons has been demonstrated in a variety of systems, but only rarely in the auditory system. In the cochlea, Hernandez et al. (2014) showed that channelrhodopsin-2 (ChR2), a widely used opsin in neuroscience research responsive to blue light, can be expressed in transgenic animals, and demonstrated successful activation of the auditory system with optically stimulation. In the CN, Shimano et al. (2013) successfully expressed ChR2 in CN neurons and showed local responses to light.

In this study, we demonstrate that optogenetic stimulation of ChR2-expressing CN neurons leads to activation of the IC and primary auditory cortex (ACtx). Of special interest is the temporal response to optical stimulation at high pulse rates, because the ChR2 ion channel has sluggish kinetics (Boyden et al., 2005), which may limit the ability to transmit fast temporal information when compared to acoustic stimulation. We argue that new approaches to the ABI using optogenetics could be explored as a means to more effectively restore hearing to ABI users, as optogenetics can potentially provide more specific activation of individual frequency regions by focusing light, and even offers the possibility of stimulating different cell types by independently using different wavelengths.
Methods

Surgical procedures and transfection of AAV2/8-ChR2 to the cochlear nucleus

All experimental procedures on CBA/CaJ mice were performed in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals as well as approved animal care and use protocols at the Massachusetts Eye & Ear Infirmary, Boston, MA. These procedures were not performed by the student and are detailed in Darrow et al. (2014). In short, 27 normal hearing CBA/CaJ mice aged 8 – 12 weeks were anesthetized with xylazine and ketamine. The dorsal cochlear nucleus (DCN) was surgically exposed. In 24 of these 27 mice, 1.0 – 2.0 μL of adeno-associated virus with channelrhodopsin-2 (AAV2.8-ChR2) was pressure injected into the CN using a 10 μl Hamilton syringe. 2 sham mice underwent the same surgical protocol, including insertion of an empty Hamilton syringe. 1 additional mouse did not undergo the surgery or injection described above and was used as control. After 3 – 4 weeks to allow for expression of ChR2, mice were prepared for acute anesthetized experiments by re-exposing the DCN and creating a superior contralateral craniotomy over the IC and/or primary auditory cortex (ACtx) to allow access for the recording electrode. Animals were sacrificed at the end of an experiment and their brains processed for histological analysis.

Stimuli

Optical stimuli were produced by a laser (BL473T-100FC, Shanghai Laser & Optics Century Co.), connected to an optical fiber (400 μm diameter) with the tip directly contacting the exposed surface of the DCN. Blue light (473 nm) pulses were 1 ms in duration and presented continuously at 5 pulses/sec unless otherwise indicated. The number of pulse presentations was typically 80, but varied from 40 to 120. For the subset of experiments characterizing the effect of pulse rate, pseudorandomized 500 ms pulse trains, followed by 500 ms with no stimulation, were presented with pulse rates ranging from 5 – 320 Hz. Light intensity was varied between −15.4 and 29.1 dB re: 1 W/cm², which adequately captured the range of firing rate responses from threshold to saturation. The laser was calibrated by positioning the optical fiber 2 mm from a high-sensitivity thermopile sensor (Coherent PS19Q) connected to a power meter (Coherent LabMax-TOP). The voltage command parameters were systematically varied, and the measured power was divided by the cross-sectional area of the fiber and by the pulse rate to get the radiant exposure in mJ/cm².

Multiunit recordings and spike detection

Multiunit recordings were performed in the IC using a 16-channel linear silicon probe, or in ACtx using a 4x4 shank array (177 μm² contact area, 50 μm contact separation, NeuroNexus Technologies). Recording probe position was adjusted to obtain a complete tonotopic map across electrodes in response
to acoustic tone pips (11.3 – 45.25 kHz in 0.5 octave steps, 0 – 80 dB in 10 dB steps, 20 ms tone duration, 10 presentations/s).

Spikes were detected from the voltage waveforms of multiunit activity similarly to Chapter 2 (see Methods): After amplification of the waveforms, subtraction of the across-electrode average signal, and filtering between 500 and 3000 Hz, spikes were defined as waveform segments that were greater than 4 times the background noise level after filtering. For pulse rates of 5 Hz, the background noise level was defined as the median value of the filtered waveform between 12 ms post-stimulus onset and the onset of the following pulse (because optically evoked spiking was never observed at a time longer than 12 ms post-stimulus onset). For pulse rates higher than 5 Hz, the background noise level was defined as the median value of the filtered waveform during the period between pulse trains.

Data analysis

Average firing rate response: To compute average firing rate, spike count was determined in a set analysis window and divided by window duration. For 5 Hz pulse trains, the analysis window was 1-12 ms post-stimulus onset. For higher pulse rates, the analysis was completed during the 500 ms stimulation period. Rate/intensity function (RIF), activation threshold, and activation bandwidth were defined and computed as described in chapter 2.

Temporal response: To investigate temporal properties of laser-evoked spiking, we computed the synchronization index (SI; Goldberg and Brown, 1969) and evaluated statistical significance with a Rayleigh test of uniformity (Mardia and Jupp, 1999). The analysis window for the SI was the entire stimulus cycle as any truncation would introduce a bias. Synchronization index/intensity function (SIF), synchronization threshold, and synchronization bandwidth were defined and computed as described in chapter 2.

Results

27 mice were used for these experiments and prepared as described in the Methods. Of the 24 mice that received an injection of ChR2, 18 had successful transfection as shown on histology (ChR2+ cases) while 6 other cases showed no sign of transfection in the CN (ChR2-). Details of the histological analysis are available in Darrow et al. (2014).

Average firing rate response in the inferior colliculus

We measured multiunit activity in the IC in response to blue light stimulation of ChR2 transfected CN neurons in mice. Figure 3.1 shows the average firing rate response in an example mouse (KND23, ChR2+ on histology). The stimulus was a 5 Hz continuous light pulse (1 ms pulse width) repeated 120
times and presented for a range of light intensities. The dot raster from IC electrode #8 (Fig. 3.1A) shows no activity other than spontaneous spiking at low light intensity, but there is an increase in spiking near stimulus onset for intensities as low as 11.3 dB re: 1 W/m². This activation becomes clearer at 20.2 dB re: 1 W/m². When plotting average firing rate between 1 – 12 ms in this electrode as a function of light intensity, the resulting RIF has a sigmoidal shape with a threshold near 6 dB re: 1 W/m² and saturation near 20 dB re: 1 W/m² (Fig. 3.1B, blue line). When repeating the same procedure with the laser in the
OFF position, the resulting RIF (shown in black) is flat with an average firing rate near 40 sp/s. Across electrodes (Fig. 3.1C), activation in the laser ON condition was quasi-homogeneous, with a significant increase in activity at the highest light intensities and quick saturation. Plotting activation threshold as a function of electrode (Fig. 3.1D) reveals that thresholds were similar across electrodes (ranging from about 6 – 11 dB re: 1 W/m²) when the laser was ON. Activation threshold was not crossed when the laser was OFF (black symbols), suggesting that the significant activation in the ON condition is a result of neural stimulation by the laser blue light rather than a consequence of an electrical artifact of our setup.

Additional experiments in other animals were done to control for an optophonic artifact. In particular, IC activity in ChR2+ cases was measured with the laser pointing away from the animal and the laser in the ear canal. No significant increase in activity was detected in these cases. In addition, no responses were measured in sham, control, or ChrR2- mice.

Figure 3.2 shows the effect of increasing pulse rate on average firing rate response in another ChR2+ example case (KND37). For all light intensities that elicited a significant response, there was a monotonic relationship between stimulation pulse rate and average firing rate, consistent with the increased total stimulation energy for higher stimulation rates.

Temporal response in the inferior colliculus

The temporal response to blue light stimulation of the CN was assessed with the synchronization index (SI). In the same animal as Figure 3.1, a peristimulus time histogram (Fig. 3.3A) shows phase-locking to stimulation pulse rate at light intensities above activation threshold. The plot of SI as a function of light intensity in an electrode (Fig 3.3B) shows a monotonic increase when the laser is ON.
while the SIF is flat with no significant synchronization when the laser is OFF. The SIF with the laser ON has a threshold near 10 dB re: 1 W/m² and saturates quickly around 25 dB re: 1 W/m². Across electrodes, the SIF profile is fairly homogeneous (Fig. 3.3C) with thresholds in the 10 – 15 dB re: 1 W/m² range (Fig. 3.3D). No significant synchronization was seen across electrodes when the laser was OFF (black symbols).

The effect of pulse rate on SI is illustrated in another ChR2+ case (KND37) in Figure 3.4. For light intensities that elicited a significant firing rate response, the SI/pulse rate functions tended to have a bandpass shape, with an increase of SI at moderate pulse rates, and a roll off at the highest pulse rates, above 33 Hz. This high pulse rate roll-off is consistent with the sluggish kinetics of ChR2 (Boyden et al., 2005).

**Figure 3.3:** Example IC synchronization response in a ChR2+ case (KND23). A. A clear response near stimulus onset at the largest stimulus intensities is observed in poststimulus time histograms (5000 bins, electrode #8, 5 Hz pulse rate, 1-ms duration light pulses). B. Synchronization index (SI) in same electrode (blue dots are means, error bars show ± 2 standard deviations, blue line: sigmoidal fit). The SI threshold is represented by a blue diamond. Black symbols show laser OFF condition. C. SI as a function of intensity and electrode. D. Synchronization thresholds as a function of IC electrode.
Effects of stimulation pulse rate on blue light responses

While Figures 3.2 and 3.4 show effects of stimulation pulse rates on blue light responses in an example mouse for a range of pulse rates, we systematically studied these effects by comparing a low pulse rate condition (5 Hz) to a high pulse rate condition (320 Hz). Contrary to electrical stimulation, which produces a large stimulus artifact that biases average firing rate and SI metrics at high pulse rates (see Chapter 2), optogenetic stimulation of the CN does not produce an artifact. Therefore our estimates of average firing rate and SI at high pulse rates are reliable.

Figure 3.5 shows the comparison of these two conditions across all ChR2+ mice and all IC recording electrodes. The maximum firing rate of the IC response (or plateau of the RIF) was significantly larger in the high pulse rate condition compared to the low pulse rate condition (Fig. 3.5A, left panel; p < 0.001 with Mann-Whitney U-test). This is consistent with the increased stimulation energy produced by delivering more light pulses per unit time. There was also a significant effect of pulse rate on maximum SI. SI in the high pulse rate condition was significantly lower than in the low pulse rate condition (right panel; p < 0.001 with Mann-Whitney U-test). In fact, in most electrodes of most mice, spike trains were not significantly phase-locked to the stimulation pulse trains (p > 0.01 in Rayleigh test of uniformity). In summary the high pulse rate condition increased average firing rate at the expense of temporal precision.

Surprisingly, increasing pulse rate did not significantly affect activation thresholds (Fig. 3.5B, left panel; p = 0.14 with Mann-Whitney U-test), in spite of the increased stimulation energy of the high pulse rate condition. There was a significant increase in synchronization threshold at high pulse rates (right panel; p < 0.001 with Mann-Whitney U-test), consistent with the numerous cases that did not exhibit any significant phase-locking at 320 Hz.

Figure 3.4: Effect of stimulation pulse rate on IC synchronization response in an example (KND37) across different light intensities. Electrode #14 is represented. At the higher light intensities, SI rolls off at pulse rates above 60 Hz.
Activation width was not significantly different between the two pulse rate conditions (data not shown). The spatial pattern of IC responses was usually broad (see examples of Fig. 3.1C-D, Fig. 3.6A), although some cases showed variations across electrodes (Fig. 6A, top). Across all ChR2+ cases, the mean activation width was 11.8 electrodes (standard deviation: 5.7). Mean activation width in ChR2- and control cases was 0.2 (standard deviation: 0.4).

Optogenetic activation of the CN activates primary auditory cortex

While most of our data was recorded in the IC with a linear electrode array, we also measured responses to CN light stimulation in primary auditory cortex (ACtx) in 2 animals with a 4x4 array. Figure 3.6 shows the average firing rate and SI responses in one of these cases, measured both in the IC and ACtx. In this example, there was broad activation of the IC (Fig. 3.6A) with overall poor synchronization.
that improves slightly at high light intensities (Fig. 3.6B). Responses in ACtx were overall similar to the IC. Average firing rate in ACtx (Fig. 3.6C) showed broad activation, with somewhat more heterogeneity across electrodes and slightly lower firing rates than IC. Thresholds were 10 – 20 dB re: 1W/cm² range, similar to IC thresholds. SI map in ACtx (Fig. 3.6D) showed overall poor synchronization with somewhat increased SI at high light intensities. Of note, some of the heterogeneity seen in ACtx may be due to the arrangement of electrodes, as the 4x4 array groups electrodes by 4 along a cortical column. This activation of ACtx shows that blue light stimulation of the CN successfully activates the higher stages of the auditory system, which suggests that optogenetic control of the brainstem may be successful in

Figure 3.6: Example response to optical stimulation of the CN (KND25) throughout the auditory pathway in the IC (A-B) and the auditory cortex (C-D). Both firing rate responses (A-C) and SI (B-D) are represented. The linear 16-electrode recording array was parallel to the tonotopic axis of the IC. The cortex recording electrode array was in a 4x4 configuration, with each group of 4 electrodes located in a cortical column and represented by brackets.
producing auditory percepts in a future optogenetic neuroprosthesis.

Comparison of optically- and acoustically-evoked IC responses

In previous sections, we demonstrated that optical stimulation of the CN with periodic pulses produces a synchronized increase in firing rate in the IC and ACtx. Figure 3.7 shows the average firing rate and temporal responses measured in the IC of an example case (KND21) in response to acoustic and optical stimulation. The acoustic stimulus was a 23 Hz continuous pulse train with pulse width 0.1 ms, while the optical stimulus was a 5 Hz pulse train with 1 ms pulse width. In this example, the optically-evoked response is consistent with the trend described previously, in that there is a significant increase in

Figure 3.7: Comparison between optical (A-B) and acoustic (C-D) stimulation in a ChR2+ example mouse (KND21). Both firing rate (A-C) and SI (B-D) responses are shown. Stimuli were different in the two stimulation modes (1 ms duration blue light pulses at 5 Hz vs. 0.1 ms duration acoustic clicks presented at 23 Hz).
firing rate broadly distributed across IC electrodes (Fig. 3.7A), with moderate synchronization (Fig. 3.7B). The acoustically-evoked response is what one would expect in the IC: Because of its broad frequency spectrum, an acoustic click excites neurons of all CFs, and the resulting firing rate map shows broad activation at similar thresholds across electrodes (Fig. 3.7C). There was good synchronization to the acoustic stimulus with SI reaching about 0.6 in most electrodes (Fig. 3.7D).

The pattern of optically-evoked response is overall very similar to that of the acoustically evoked response (broad activation with a monotonic increase in firing rate and SI). The multiunit firing rates obtained in both modes of stimulation were comparable (maxima near 400 sp/s in the optical case, vs 500 sp/s in the acoustic case). Of note SI was on average lower in the optical case than in the acoustic case.

Discussion

In this study, we demonstrate that optogenetic control of ChR2 infected CN neurons of mice produces synchronized increased in activity throughout the auditory system (IC and ACtx). The measured responses were comparable to that of an acoustic click delivered near the tympanic membrane.

Optogenetic stimulation of the CN elicits broad activation

The activation bandwidth of IC and ACtx optically-evoked responses was characteristically broad (see examples of Fig. 3.1C-D, Fig. 3.6A-C, Fig. 3.7A), consistent with activation of a large portion of the tonotopic axis. The IC rate response was in effect similar to that of an acoustic click (Fig. 3.7A-C), an acoustic stimulus that has a wide spectrum that should excite neurons of all CFs. One of the motivations for using optogenetics for CN stimulation rather than electrical stimulation was the hope of producing a narrow response in the frequency domain, as a result of potentially improved spatial selectivity of optical stimulation. Our results indicate that in our experiments, the spatial selectivity of light stimulation was not appropriate for obtaining the type of frequency resolution necessary for understanding speech. This poor spatial selectivity in our experiments was in fact expected from our experimental setup, as the diameter of the optical fiber used for stimulation of the CN was 400 μm, which is close to the dimension of the DCN, therefore spanning the entire tonotopic axis of the DCN. Future experiments with fibers of smaller dimension or devices to focus the light will be necessary to truly test whether light can provide better spatial selectivity.

Temporal response to ChR2 optical stimulation

The temporal response assessed by SI in the IC and ACtx shows the ability of the auditory system to phase-lock to optically-evoked light pulses delivered to ChR2+ CN neurons. While the SI achieved in low pulse rates condition were consistent with moderate synchronization to the stimulation pulse trains,
fast stimulation rates strongly degraded SI in most cases (see summary data in Fig. 3.5A, right panel). The comparison in an example between optically-evoked and acoustically-evoked SI (Fig. 3.7B-D) shows lower SI in the optical case than in the acoustic case. One of the issues with this comparison is that the pulse rate in the optical condition was lower (5 Hz) than in the acoustic condition (23 Hz). Further, pulse widths were different between the two conditions, giving a synchronization “advantage” to the acoustic condition, which had a shorter pulse width (0.1 ms in the acoustic case, 1 ms in the optical case).

Another contributor to the roll-off of SI at high pulse rates we observed may be the somewhat sluggish kinetics of ChR2. In select examples where we measured responses with a range of pulse rates, we observe this roll-off near a pulse rate of 60 Hz (see example of Fig. 3.4). This cutoff is consistent with the kinetics of ChR2 (Boyden et al., 2005; Ishizuka et al., 2006; Lin et al., 2009). As temporal information is important for speech understanding (Shannon et al., 1995; 1998), the overall moderate SI we found at most pulse rates, with a significant breakdown at higher pulse rates, may be an issue with the ChR2 opsin. It may be necessary to use different opsins (e.g. ChIEF, ChETA, Chronos) to convey meaningful temporal information to the human CN as these new opsins have faster kinetics (Lin et al., 2009; Gunaydin et al., 2010; Klapoetke et al., 2014).

In addition to the sluggish ChR2 kinetics, another factor that may have limited our ability to detect higher synchronization at higher pulse rates is the fact that we recorded multiunit rather than single unit activity. Even with high SI in individual units, one would expect that the SI in multiunit activity would be lower because of different synchronization phases across individual units. This effect should be less prominent at lower rates, when firing phases are small compared to the period of the stimulus.

Toward an optogenetic ABI?

It is encouraging that we were able to measure responses that were physiologically similar to the responses to an acoustic click in various stages of the auditory system, including ACtx. This suggests that an unanesthetized animal would have experienced an auditory percept with optical stimulation. One of the aims of this study is to lay the groundwork towards developing a new generation ABI that might provide better frequency resolution than current electrical technology, although we did not test this hypothesis in this study. This hypothesis seems to hold in the cochlea, where Hernandez et al. (2014) showed better frequency resolution with optical stimulation than with monopolar electrical stimulation.

Another interesting feature of optogenetics that could potentially be advantageous over electrical stimulation is the fact that opsins can be selectively expressed in different cell-types. This would allow an optogenetic ABI to selectively activate different populations of neurons, and therefore better imitate normal physiology rather than drive all CN cell types with the same stimulus. Although the ABI is placed on the surface of the DCN, deeper areas of the DCN and other subdivisions of the CN (antero-ventral and
postero-ventral CN) may be activated as well from penetration of light through the neural tissue.

An important limitation to the development of optogenetic ABIs is the use of a viral vector to deliver ChR2. In humans, this technique has been used in gene therapy trials, but the field has been under heightened scrutiny ever since the death of a patient enrolled in one such trial in the late 1990s. Yet, more recent clinical trials using adenoviruses as vectors are promising not only in terms of safety but also efficacy (e.g. MacLaren et al., 2014). A few commercial drugs using gene therapy are currently approved in China (Gendicine, a recombinant adenovirus with wild-type p53 used to treat some types of cancer) and Europe (Alipogene tiparvovec, uses an adenovirus vector to treat lipoprotein lipase deficiency). Further progress in gene therapy alongside the technological developments necessary to build an optically-based neuroprosthesis will be needed to translate these techniques to human subjects.
In Chapter 2, we showed that electrical stimulation of the surface of the DCN of rats does not provide adequate frequency resolution, therefore partly explaining the difficulty ABI users have in understanding speech. Stimulation location on the surface of the DCN had minor effects on responses. Increasing stimulation pulse rate increased firing rate response at the expense of temporal precision. A novel, flexible polyimide-based microelectrode array elicited well-synchronized responses with low activation thresholds. Co-stimulation with infrared light produced an inhibitory response in one animal. In Chapter 3, we demonstrated the possibility of using optogenetics to stimulate the DCN of mice and successfully activate the central auditory nervous system. Although the activity measured in the IC in response to light pulses was similar to the activity elicited by acoustic clicks, the temporal response of optically-evoked activity was only moderately synchronized, and further degraded at pulse rates above 60 Hz. In this chapter, we provide a general discussion of the aforementioned findings, with a focus on future work to further elucidate these questions.

Development of flexible electrode microarrays

One of the aims of this project was to test in vivo a new flexible microelectrode array manufactured by our collaborating team at EPFL (Chapter 2). The design we tested included 2 microelectrode contacts mounted on a flexible polyimide base. The rigidity of currently approved human ABIs does not allow the array to conform to the surface of the curved CN. It is possible that the spread of electrical stimulation is exacerbated by the non-optimal contact of the neuroprosthesis to the neural tissue. We did not specifically test for this hypothesis in our study. It will be important to formally test this by comparing the activation width of CN stimulation in flexible arrays against rigid arrays of otherwise similar geometry and impedance profiles. Different materials can be used to simulate a rigid ABI, such as silicone, and EPFL has the capability to build such arrays. The degree to which the array is flexible can also be modulated by changing the extent to which the polyimide flexible material is perforated. To increase the validity of these comparisons, trying arrays of various rigidities in the same animals will be key.

Electrode design could also be modified to allow for a more controlled way of studying the effect of stimulation location on the response. In our experiments, we studied these effects by pseudo-randomly positioning our smaller “twisted” electrode onto one of 12 locations on the surface of the exposed DCN. Comparison across locations is difficult because a full location study necessitates repositioning the electrode 11 times, which tends to injure the neural tissue. Moreover, the angle of contact between the
bipolar electrode and the neural tissue, as well as the pressure of the electrode on the tissue, is not the same across all locations and cannot be easily controlled. Therefore, the differences in response that we measured across stimulation location need to be taken with a grain of salt. The EPFL electrode could be modified to address some of these concerns. In particular, a design with a rectangular array of 10 or more electrodes, spanning the entire surface of the DCN, would allow the investigator to study the effect of location after positioning the electrode array only once on the DCN surface. Effects of the distance between the 2 electrodes contacts used for bipolar stimulation could also be investigated with this design.

Exploration of INS-electrical co-stimulation

In one animal, we were able to stimulate the surface of the CN conjointly with electrical and IR pulses (Chapter 2). Our stimulation paradigm consisted of a 2 ms IR pulse immediately followed by a 200 µs biphasic electrical pulse presented at a rate of 23 Hz. Our measurements in the IC were consistent with an inhibitory effect of INS, as both threshold and absolute firing rate decreased with co-stimulation as compared to electrical stimulation alone. We suggested that if INS inhibits CN neurons (or excites preferentially inhibitory neurons), an ABI design with electrodes flanked by IR optodes could, in theory, help improve spatial selectivity and therefore the frequency resolution achieved with electrical stimulation. We point to difficulties in translating this opto-electronic ABI into a clinical application in the Discussion section of Chapter 2.

It is important to emphasize that these results were obtained in one animal and need to be replicated. The experiment is difficult to perform because the animal needs to be deafened to avoid any bias from the well described optophonic artifact of the IR laser (Verma et al., 2014), and this was achieved by transecting the AN. In addition, positioning the optical fiber next to the stimulating twisted electrode was extremely difficult due to the small size of the DCN and restricted access through the posterior craniotomy. Using the flexible array designed by EPFL might help in studying this phenomenon. In particular, one possibility would be to intersperse electrodes and optodes on the array, which would allow the investigator to set up both stimulation modes at once, and study different spatial configurations of co-stimulation.

In addition to replicating the results, exploring the vast multi-dimensional parameter space of IR-electrical co-stimulation is crucial. In data not presented in the report, we tried different delays between the IR pulse and the electrical pulse (including having the electrical pulse presented first) and different pulse durations. The paradigm we adopted seemed to elicit the most reliable effect in this one animal. However, this study was preliminary, and investigating the effects of delay and pulse width in more details, along with effects of IR light intensity, pulse rate, stimulation location, distance between IR laser and electrode, among other parameters, is important.
If this effect of IR light on CN neurons is confirmed, understanding it will be key to determine whether it can be translated into a clinical application. One important question is whether the observed effect is a result of IR stimulation per se, or of an increase in temperature of the stimulated neurons.

Comparing the neurophysiologic responses to optogenetic and electrical stimulation

The spatial selectivity of optogenetic stimulation cannot be directly compared to that of electrical stimulation from the data of these two studies. One of the issues making such a comparison hazardous is the fact that the data was obtained from different species. Although one of the motivations for using optogenetics is the potential for focusing the light and stimulating a narrower region of the CN, we did not directly test this possibility. Rather, we limited our effort to demonstrating the feasibility of optogenetic stimulation of the CN. Our results point to very broad activation of the tonotopic axis with our setup, which was not different from our electrical stimulation results. It is however important to note that in the case of electrical stimulation, we stimulated discrete locations of the DCN surface, whereas the optical fiber we used to stimulate the mouse DCN was almost as large as the DCN surface itself. Therefore it is not surprising that spatial selectivity was poor in the optical case. To test whether the poor spatial selectivity found in our study can be improved, once could use a laser collimator to focus the light onto a narrow region of the CN, and compare between IC responses with the current setup and IC responses obtained with collimated blue light.

Future studies should try and directly compare electrical and optogenetic stimulation. To do so, the EPFL electrode design could be modified to include small stimulating electrodes positioned close to blue light optodes of similar size on the array. The IC responses could then be compared in the same animal and for nearby stimulation location, therefore controlling for stimulation location. It will also be important to compare the temporal responses obtained in both cases, as our study suggests poorer synchronization with optogenetics than with electrical stimulation, although the many differences between studies do not allow drawing definite conclusions.

Comparing the behaviors elicited by optogenetic and electrical stimulation

In this report, the study of the spatial selectivity of CN stimulation was motivated by the assumption that this selectivity was a predictor of frequency resolution. From a neurophysiologic standpoint, there is a correspondence between the two concepts, because of the tonotopic organization of the auditory system, which more or less maps location along certain axes onto spectral frequencies that are most excitatory to auditory neurons. Yet, it is important to test whether any improvement in spatial selectivity translates into improved frequency resolution at a perceptual level. Current work is being done in our laboratory to study the frequency resolution of optical stimulation in awake, behaving mice with a
chronically implanted optical fiber. This work should be extended to study awake, behaving mice with the chronically implanted opto-electronic array described in the previous section, in order to directly compare their performance in a frequency discrimination task between optical and electrical conditions. A gap detection paradigm could also be used to compare the performance of the behaving mice in a task relying primarily on temporal information.

Overall, these experiments will provide insights into both the neural encoding of artificially delivered stimuli and their effects on behavior. The proposed direct comparison between electrical and optical stimulation strategies may help the development of new generation ABIIs and neuroprostheses.
References


