Responses from electric stimulation of cochlear nucleus

by

Ryuji Suzuki

Submitted to
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Abstract

Cochlear nucleus (CN), the exclusive destination of the auditory nerve, is the gateway for all central processing of auditory information. The CN comprises three major subdivisions: anteroventral, posteroventral and dorsal (AVCN, PVCN and DCN, respectively), each of which contains anatomically and physiologically distinct neurons projecting onto different targets. This research used focal electric stimulation of small, confined parts of the CN in anesthetized guinea pigs to resolve the roles of the CN divisions, in two contexts. Part 1 explored the effect of stimulation on the gross neural potential (electrically evoked auditory brainstem response, EABR). In AVCN and PVCN away from the 8th nerve fibers entering the brainstem, stimulation consistently evoked waveforms comprising 3 waves, suggesting a diffuse distribution of cellular generator of the EABR. On the other hand, in vestibular structures (vestibular nerve root and Scarpa's ganglion), the characteristic waveform comprised only two waves. Stimulation of multiple neural structures, as seen with higher stimulus levels or stimulation in auditory nerve root area generally produced more complex and variable waveforms. Part 2 explored the effects of stimulation on the activation of one type of auditory reflex, medial olivocochlear (MOC) reflex. The reflex was monitored through its effects on distortion product otoacoustic emission (DPOAE). The MOC reflex was activated bilaterally by stimulating PVCN or AVCN shell, but not AVCN core. These results suggest that there are two groups of MOC interneurons in specific parts of CN.

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Chapter 1

Overview

1.1 Introduction

The CN is the gateway to the central auditory system. The auditory nerve inputs drive multiple cell types in different subdivisions (anteroventral, posteroverentral, dorsal) of the CN, with each cell type in turn projecting centrally to different targets. Thus CN is the origin of the parallel pathways in the central auditory system. All parallel pathways are presumably activated by sound stimulation, but we are ignorant of the functional significance of most of these pathways in the overall hearing experience. The cochlear nucleus (CN) neurons and their output pathways have been known, mostly in terms of anatomical projections and single unit responses to acoustic stimuli. Our approach is to investigate the responses from selectively stimulating a sub-set of the CN-originated parallel pathways. Examples of previous research using this approach are sparse; most of them are related to study of auditory brainstem implant. Some studies (Oda et al. 2009) studied particular design of stimulating electrodes, whereas others studied evoked responses obtained from patients who received ABI. However, a careful study of basic physiological responses from focal electric stimulation of CN is lacking.

In this research, a focal electric stimulation was made using small electrodes and low stimulus levels, so that we can characterize the neural responses in relation to the place of stimulation within CN. The response was recorded in two gross physiological measures: electrically-evoked auditory brainstem response (EABR; chapter 2), and distortion product otoacoustic emission (DPOAE; chapter 3).

In the EABR study, the response from the CN neurons and their target pathways was measured in the form of gross evoked potential, with reference to the ear ipsilateral to the CN being stimulated, while a part of the CN is focally stimulated (Figure 1). Stimulated CN cells would typically respond by generating an action potential, and when a sufficient number (a few hundreds or more) of cells are recruited, this can be measured as a compound action potential. Responses at downstream targets may similarly contribute to delayed compound action potentials. Therefore, EABR may
Figure 1. The CN, parallel pathways and the experimental paradigm of the EABR study (Chapter 2). The CN is indicated by yellow shading. The gross activity of the auditory pathway can be recorded at the vertex with respect to the ipsilateral (ipsi) ear as voltage waveform. AVCN-a: anterior part of the AVCN. AVCN-p: posterior part of the AVCN. PVCN-a: anterior part of the PVCN. PVCN-p: posterior part of PVCN. Figure adapted from Melcher and Kiang (1996).

reflect neural activities related to the specific CN neurons being stimulated.

The measurement of EABR is similar to acoustic ABR, typically evoked by clicks or chirps, rather than electric pulses. Sound stimulates several types of cells in the CN, but only a few of them are known to contribute to the ABR (Melcher et al. 1996a, 1996b; Melcher and Kiang 1996), due to such factors as cell axon diameter, cell counts, and synchronous firing patterns. Cellular generators of ABR are generally known (Melcher et al. 1996a, 1996b; Melcher and Kiang 1996) and they are good candidates for cellular generators of EABR. However, with electric stimulation, synchrony is near perfect, but the distance to the stimulating electrode is now a factor. The result of the EABR study suggests that bushy cells are most likely generating the first wave with stimulation within the core of the CN. Vestibular and auditory branches of the 8th cranial nerve are also capable of generating gross evoked potentials, and this may contaminate or obscure the response from the CN core if stimulus level is too high or
if the stimulus site is near the 8th nerve entering the brainstem.

There is increasing interest in investigating the response to electric stimulation, for basic scientific purposes and to improve the neural prosthesis that stimulates the CN, the auditory brainstem implant (ABI). Insight gained from the EABR response might be found useful in understanding and improving the current generation ABI technology.

The MOC reflex is thought to reduce the masking effects of background noise (Winslow and Sachs 1987; Kawase et al. 1993) and protect the cochlea from acoustic overstimulation (Rajan 1988; Reiter and Liberman 1995). The MOC neurons are located in periolivary regions of the superior olive and project to the cochlea, where they influence otoacoustic emissions. In Chapter 3, distortion product otoacoustic emission (DPOAE) was measured as an assay for activation of medial olivocochlear (MOC) reflex. The interneurons for this pathway are anatomically identified in the cochlear nucleus through double labeling studies (Robertson and Winter 1988, Thompson and Thompson 1991, Ye et al. 2000), which indicated locations in two different subdivisions of the CN. Our studies may indicate which subdivision contains neurons that are capable of activating the MOC reflex using a physiologic test.

The MOC reflex pathway has rather few thin axons and likely does not contribute to the gross far field potential, thereby rendering itself invisible from an EABR measurement. DPOAE measure is a much more sensitive assay for the MOC reflex pathway. On the other hand, the EABR is likely to include responses from all stimulated pathways, as long as their activation timing is synchronized, and there are a sufficient number of thick axons running together. Thus, these two measures are complementary regarding the type of information they may provide.

1.2 Methods: detailed addendum

Basic information about the experimental methods are explained in Chapters 2 and 3. In this section, details are expanded for techniques I developed or improved for these studies.

1.2.1 Fixative and staining of the stimulation sites Site of stimulation was stained with Prussian blue pigment. This procedure consists of two steps: (1) deposition of ferric ion by passing DC current, and (2) reacting ferric ion with hexacyanoferrate (II). During step 1, stainless steel electrode containing iron (o) is oxidized to iron (III) at the anodic. A 0.1 mA DC current was passed over 10 to 30 seconds, for a total charge of 1 to 3 mC, for an electrode of 400 µm. If the electrode is in good contact with the tissue, low end of this range is expected to be adequate, but in some cases, high end of this range did not result in convincingly dark staining. This is probably due to poor tissue contact—if the electrode contact is loose and there is a CSF-filled space, the ferric ion may be lost during processing.

The fixative solution contains potassium hexacyanoferrate (II), which reacts with ferric ion to form Prussian blue pigment. The ferric ion, however, is also reactive with hydroxide ions, and this is in competition with the Prussian blue reaction.
Therefore, it is imperative that the pH of the fixative does not exceed 7.4. In fact, adjusting the fixative pH to 6.8 would be advantageous.

The best technique I found was to flood the dorsal surface of the brainstem with a few drops of 10% solution of potassium hexacyanoferrate (II) to form insoluble Prussian blue pigment, at the moment the animal is given an overdose of urethane, but prior to perfusion or immersion fixation. This technique tends to form darker but confined staining, since diffusible ferric ion is quickly converted into insoluble pigment. The fixative for this process needs not contain hexacyanoferrate (II), and pH is not critical.

1.2.2 Recording electrodes EABR signal was recorded with a silver wire placed between the dura and the skull, slightly off the midline of the vertex. Silver wire was slightly better than stainless steel screw or wire previously used, but it was by no means ideal, due to large flicker noise (1/frequency noise or pink noise) generated at the liquid-metal junction. This low frequency noise component is larger than neural signal of interest, but it could saturate the amplifier in a time-varying manner. People who follow this technique should invest some time to investigate other electrode materials that generate less flicker noise.

1.2.3 EABR recording The signal was amplified with minimal of filtration and sampled at 80 kHz. Conventional ABR recording typically use 300 to 3000 Hz bandwidth, since such bandwidth is adequate to represent signal of interest and enhances signal-to-noise ratio. However, filtration in EABR recording will make the artifact last longer, since the actual signal artifact is convolved with the impulse response of the filter before the signal is observed. The same applies to the antialiasing filter typically used with digital-to-analog (D/A) converter and analog-to-digital (A/D) converter. It was crucial to use A/D converters without antialiasing filters for EABR recording.

One large component of the artifact was a time constant formed by the impedance of the recording electrode and the capacitance of the amplifier's input cable. In one experiment, Ithaco amplifier was replaced with a small custom amplifier placed immediately next to the experimental animal apparatus, so that this capacitance was minimized, and the artifact was significantly reduced by this change alone.

A significant component of the artifact was generated within the amplifier due to saturation. Once saturated, the amplifier takes a finite duration of time to recover and even longer time to arrive at the stable amplification gain and baseline. Although Ithaco amplifier was a better option from what was available in the lab, a better amplifier could be inexpensively custom built using modern analog electronics technology. Such an amplifier can offer wider dynamic range and faster recovery from clipping.

Grounding of the animal also had a significant effect in artifact reduction. With poor choice of animal grounding, the amplifier can be saturated in the common mode before differential mode. This is undesirable but also avoidable by grounding the animal at a place about halfway between the vertex electrode and the reference electrode. In the present study, the animal was grounded at the bite bar and this reduced stimulus artifact by preventing common mode saturation.
The AC power and ground lines for the analog system should be isolated from those for the rest of the system. In the present study, considerable amount of noise was generated by the PXI system (National Instruments) itself in its power supply module (frequency above 75 kHz), and it was conducted to the power ground. This noise, in turn, appeared in the ground and power lines of the sensitive analog equipment, and contaminated EABR recording. Since line conducted noise below 150 kHz is unregulated, many devices can emit considerable amount of noise, and their aliases may appear in frequency range of the signal itself. Similar problems are anticipated not only in EABR recording but whenever high bandwidth unfiltered analogue recording is needed. The experimental system’s noise immunity was enhanced to a sufficient level by use of two isolation power transformers (one on PXI system, another on the analog systems), differential transmission of the analog signal to the PXI system, and reorganizing power grounds and signal grounds.

Any future research facility intended for EABR recording should be designed with all these factors considered in the very early phase of planning.

1.2.4 EABR artifact suppression The most important element of the EABR is the first wave, which is generated by the CN neurons, but it occurs within a few hundred microseconds of the stimulus pulse, and usually is obscured by the artifact. Thus, any meaningful analysis would require near-perfect removal of the artifact. Since the goal of the study necessitated to design the stimulus parameters so that only most sensitive neurons nearby are excited, monopolar electrode and monophasic pulse were chosen, the combination most challenging in terms of artifact-to-signal ratio. This was very painful but necessary price to obtain interpretable results.

A practical artifact suppression technique was developed using a passive linear model of artifact generation.

In the block diagram in Figure 2, the only observable signal is $x(t)$, and the only freely controllable component is $h_A(t)$. However, separating the components...
and denoting each with a variable allows some algebraic manipulations, where each signal is represented by a vector:

\[ y = x - \hat{h}_A \]
\[ = h_N + n + (h_A - \hat{h}_A) \]  \hspace{1cm} (1.1)

\[ x - \hat{h}_A - h_N - n = h_A - \hat{h}_A \]
\[ ||x - \hat{h}_A|| + ||h_N|| + ||n|| \geq ||h_A - \hat{h}_A|| \]  \hspace{1cm} (1.2)

where (2) was the result of triangular inequality. Our objective is to minimize the norm of the residual artifact \( ||h_A - \hat{h}_A|| \), which can be achieved by steering \( \hat{h}_A \) so as to minimize \( ||x - \hat{h}_A|| \).

The general characterization of the artifact response was obtained with several resistor networks, a beaker filled with saline with immersed electrode, and post-mortem animals, and it was adequately modeled by a sum of two decaying exponentials (eqn. 1.4). Thus we arrive at an artifact estimator:

\[ \hat{h}_A = \arg\min_r ||x - r|| \]
\[ r \in \{a_1e^{-\tau_1t} + a_2e^{-\tau_2t}\} \]  \hspace{1cm} (1.3)

However, eqn. (1.2) also suggests the risk of overfitting, if \( \hat{h}_A \) is allowed to be arbitrarily close to \( x \), in which case the residual artifact can be in the same magnitude as the magnitude of the signal and noise. In order to avoid such undesirable condition, we impose a constraint derived from eqn. (1.1) as follows:

\[ x - \hat{h}_A = h_N + (h_A - \hat{h}_A) + n \]
\[ ||x - \hat{h}_A||^2 \approx ||h_N||^2 + ||h_A - \hat{h}_A||^2 \]

where the latter equation requires that \( h_N \cdot (h_A - \hat{h}_A) \approx 0 \), and the noise term \( n \) be negligible. If the signal subspace and the artifact subspace are orthogonal, or if \( h_N \) is comprised of multiple waves, and signal-to-noise ratio is favorable, this artifact estimator (eqn. 1.3) is expected to work well. An example of signal before and after artifact suppression is shown in Figure 3.

The artifact suppression was applied to each presentation of stimulus pulse and the cleaned signal segments were then averaged. This computation was batch processed in a postexperimental analysis, but in future studies, this mechanism should be implemented in the computer system used for data acquisition, so that the clean signal can be monitored during experiments.
1.2.5 Data management  It was very important to design a standard data format, so that a large volume of data could be effectively managed by a database system, and processed by custom signal analysis programs. The experiments from Chapters 2 and 3 totaled 3368 runs, and the number of waveform figures was 11832, where each figure may contain several overlapping traces for level series, rate series, etc. If traces were to be printed and organized in traditional filing system, 24 reams of paper would be used just for the waveforms, and extracting subset of the data for each analysis would be impractical. When this was realized, each experimental data was packaged into a standardized data format, and catalogued in a relational database together with essential information, such as measurement type, stimulus parameters and stimulation sites from histology. This enabled me to analyze the data from many angles, by allowing to extract data points meeting certain conditions and re-process them in a relatively short period of time.

A vast majority of the 3368 runs and 11832 figures were not included in the next two chapters, since many of them were repeat runs (stability test), ineffective sites, and runs that were later excluded based on stimulus parameters or other experimental conditions. However, keeping them in one database was of tremendous value because it allowed me to follow the context in which selected run was recorded. Also, analysis of formerly rejected data points sometimes provided new pieces to the puzzle (e.g., vestibular stimulation sites in Chapter 2).

1.3 Mistakes made

1.3.1 Bipolar electrode for EABR study  Bipolar electrode worked very well for the DPOAE study, which was conducted first. It allowed passage of relatively large stimulus currents without causing twitches. Therefore, it was naively expected to work
well for the EABR study, but it became clear that stimulation with a crude bipolar electrode (two thin insulated wires twisted and cut together to form a sharp end) produced highly variable results. In an effort to improve this situation, a small concentric stimulating electrode was used for several experiments. However, it was later realized that simultaneous presence of cathodic and anodic stimulations was the main factor complicating the results. Only after sufficient data were obtained with monopolar stimulation, it was realized that bipolar stimulation in the CN appeared to have excited different neural populations (probably bushy cells by cathodic stimulation, and auditory nerve by anodic stimulation).

1.3.2 Higher stimulus levels for EABR study  In the first few EABR experiments, large EABR waveforms that appeared similar to click-evoked ABR (except for the absence of wave P1), using relatively high stimulus levels (125 to 500 \( \mu A \)), and such waveform was seen in three experiments. Also, signal-to-noise ratio was excellent and data could be collected quickly with such a high stimulus level. These factors gave false impression that useful knowledge could be gained by such experiments. However, a vast majority of data were uninterpretable due to lack of consistent morphology. Eventually, it was realized that, among many sites that produced very different EABR waveforms at 125 \( \mu A \) or higher, EABR's of one morphology type were indeed obtained at a low stimulus level (16 to 62 \( \mu A \)).

In the hindsight, diverse waveforms seen in the early experiments were most likely due to non-specific excitation of a combination of CN cells, auditory nerve, vestibular nerve, and perhaps other neighboring structures.

1.3.3 Use of voltage controlled stimulus for DPOAE study  All DPOAE measurements were performed with bipolar electrodes driven by a voltage-controlled source. This was primarily due to the limitation of the experimental system available at that time. It is possible to calculate the current level using Ohm’s law, with a caveat that electrode impedance is somewhat variable, and uncertainty remained. A limited number of DPOAE experiments were later performed with current controlled stimulation, where the level of stimulus required to produce comparable DPOAE effects were not dissimilar to that estimated from earlier voltage-controlled experiments.

1.3.4 Simultaneous bilateral recording of DPOAE  In very early DPOAE experiments (for which histology is not available), simultaneous bilateral measurements of DPOAE were made. The DPOAE effect was generally larger in the ear ipsilateral to the CN being stimulated. However, as the experiment system was modified, this capability was lost, and later measurements of bilateral DPOAE were not simultaneous. This raised a significant concern as to the accuracy in the DPOAE effects when compared bilaterally, and this prevented meaningful and detailed analysis of bilateral comparison.

1.3.5 Use of DPOAE assay  DPOAE assay was used because it was known that DPOAE would change when MOC reflex was activated (Mountain). However, in an early experiment, it was observed that DPOAE level increased when MOC was activated,
which raised a concern, but I continued to use DPOAE assay. An explanation of this phenomenon was advanced (Deeter et al. 2009, Siegel et al. 1982), but the use of DPOAE assay limited the ways in which the data can be interpreted. In future work, SFOAE should be considered.

1.4 Notions intentionally deferred or avoided

1.4.1 Labeling waves In ABR research, waveforms are highly stereotypical within species, and therefore waves are customarily labelled as P₁, N₁, P₂, ... in animals, or I, II, III, ... in humans. In Chapter 2, I intentionally avoided labeling of waves, since, in EABR of CN stimulation, stereotypical waveform morphology had not been established. However, as other laboratories confirm the three-waveform morphology of CN stimulation, that may be a good reason to establish a nomenclature for EABR waves.

1.4.2 "EABR Threshold" It is tempting to discuss threshold of almost any stimulus-response relation. However, in Chapter 2, this term was roundly avoided, because it is nontrivial to define threshold in a way it may be practically measured. This is because, the apparent “threshold” depends on the noise from the measurement system or the number of averaging applied.

1.4.3 Wave amplitude In EABR study, wave amplitude was only measured for the first wave, and it was measured against the pre-stimulus baseline. Amplitude measurement of later waves require a definition of meaningful reference point, since low frequency EABR components, as well as baseline shift (due to flicker noise) are added to the apparent amplitude. Such measurements would be particularly problematic when the waveform reflects multiple cellular generators.

1.5 Future work

1.5.1 Cellular generators of the EABR The first wave of EABR from CN stimulation is most likely generated by globular bushy cells (GBC) in large part. GBCs have large axons and there are many GBC’s in CN. However, according to Hackney et al., GBCs are located over AVCN-p and PVCN-a in guinea pigs. This leads to a question: why PVCN stimulation produced much smaller EABR than AVCN stimulation?

1.5.2 Preparation with degenerated auditory nerve In order to confirm the three-wave morphology of CN stimulation, two attempts were made using animals where a cochlea was destroyed by surgical drill one week prior to the experiment. However, EABR responses lacked waves. This leads to two possibilities: (1) intact auditory nerve is essential for EABR described in Chapter 2, or (2) the crude operation to destroy cochlea also affected the physiology of the CN. A more careful approach is needed to answer this question.

1.5.3 Reference electrode location The location of reference electrode was critically important in the EABR study. Like in most ABR recordings, reference electrode
was placed at the ipsilateral ear. With anodic pulses, a negative first wave was ob-
served, presumably generated by antidromic action potential of the auditory nerve. The amplitude of this negative wave was rather sensitive to the location of the refer-
ence electrode. This sensitivity of the reference electrode location could be used to an advantage in future experiments.

During the EABR study, there were a few recordings where the recording elec-
trode was placed in inferior colliculus (IC) contralateral to the CN being stimulated, as an attempt to identify waves generated by the IC or nearby structures. One step further, both recording and reference electrodes could be placed within the brainstem for more specific detection of certain waves.

1.5.4 Targeted use of noise In a few EABR experiments, loud acoustic noise was used to see whether the EABR waveforms were affected by sound. Since this was originally meant to be a “quick check” during experiments to get some assurance of stimulating auditory neurons, a pre-generated noise was played with a music player connected to a pair of amplified speakers. Ironically, the noise effect was recognized only after post-experimental processing to suppress artifact. However, since the effect was significant, it is probably worthwhile using more targeted acoustic stimuli, such as band-pass noise, or noise in the contralateral ear only.

1.5.5 Numerical modeling Once basic understanding of EABR from focal stimu-
lation in CN is established, this topic is a prime candidate for numerical modeling study. Construction of numerical models of cellular generators of EABR may open a new way to interpret EABR’s obtained from such conditions that produce currently uninterpretable data: high stimulus levels, bipolar stimulation and biphasic pulses. This is of particular importance, since chronically implanted electrodes are driven by charge balanced stimulus waveforms.

By the analytical approach, a pool of EABR waveforms may be subjected to principal component analysis (PCA) or similar analyses to represent each EABR wave-
form by a point in an abstract space, which may be useful in organizing waveform morphology. Similarly, waveforms may be subjected to one of many statistical or knowledge-based classification techniques for automatic classification of the EABR waveform type.

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Bibliography


Overview


Chapter 2

Electrically evoked auditory brainstem response from focal stimulation of cochlear nucleus

Keywords: EABR, AVCN, PVCN, bushy cells, vestibular nerve, auditory brainstem

Abstract An electrically-evoked auditory brainstem response (EABR) was recorded at the vertex while focally stimulating different parts of the cochlear nucleus (CN) in anesthetized guinea pigs. In anteroventral CN (AVCN) and posteroventral CN (PVCN) away from the 8th nerve entering the brainstem, stimulation consistently produced waveforms comprising 3 waves. Stimulus polarity had a significant effect: cathodic stimulation was four times as effective as anodic stimulation at evoking an EABR and the cathodic first wave was positive in polarity. In contrast, anodic stimulation resulted in a first EABR wave of reverse polarity, presumably reflecting antidromic firing of the auditory nerve. For cathodic stimulation, the amplitude of the first wave was largest with stimulation sites in AVCN, indicating that the primary generators of this wave are mostly located in AVCN. Complex and variable waveforms comprising more than 3 waves were seen in stimulation sites adjacent to multiple structures, e.g., sites near the 8th nerve, or at higher stimulus levels. The amplitude of all waves decreased in presence of acoustic noise, indicating that they are generated by sound-sensitive neurons. Stimulation in or near vestibular structures (vestibular nerve or Scarpa’s ganglion) produced gross potential waveform comprising only two waves.

2.1 Introduction

An electrically evoked auditory brainstem response (EABR) is a far field neural potential evoked by stimulation of auditory nerve (AN) or cochlear nucleus (CN). The existing reports of auditory nerve stimulation, mostly motivated by intraoperative monitoring and cochlear implants, show rather consistent EABR waveforms similar to acoustically evoked auditory brainstem response (ABR) except for shift along the time scale (Berryhill and Javel 2001, van den Honert and Stypulkowski 1986). On the
other hand, existing reports of CN stimulation (Waring 1996, Nevison et al. 2002, Oda et al. 2009), show widely varying results with little agreement in the morphology of waveforms.

These diverse waveforms could result from uncontrolled stimulation of the CN cells in previous studies. The CN contains multiple cell types in its different subdivisions (anteroventral, posteroverentral, and dorsal), with each type having its own characteristic axon projecting centrally to different targets. Irrespective of whether electric or acoustic stimulation is used, when the AN is stimulated, multiple cell types become activated in a stereotyped fashion. However, when electric CN stimulation is used, one or many of the cell types might become activated depending on the electrode location and the stimulus current level. Direct focal electric stimulation could be used to activate a subset of the CN cell types, and that is the approach taken in the present study. Since most cell populations are rather localized within the CN (Cant 1992), and different populations would be stimulated depending on the stimulation site and the current level, the EABR might vary depending on the site of stimulation within the CN. The present study tested whether this is the case by stimulating different CN subdivisions. The site of stimulation was verified through postexperimental histology and correlated with the waveform morphology and the first wave amplitude.

Experiments and analyses of this paper were designed for specific and focal stimulation of thick myelinated axons and cell bodies in close proximity from the stimulating electrode. A monopolar electrode was used to deliver monophasic cathodic pulses in low current levels, condition which is generally considered preferable for such goal (Ranck 1975). Furthermore, narrow pulses stimulate thicker axons more preferentially than wider pulses, wherein stimulus charge is held fixed (Ranck 1975). These choices minimize the risk of contaminating the EABR recordings intended to reflect only CN-originated signals, where extraneous neurons in distant locations might be excited and generate spurious gross potentials.

2.2 Methods

2.2.1 Animal preparation All experimental procedures on animals were in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals, and were performed under approved protocols at the Massachusetts Eye and Ear Infirmary. Experiments were performed with 37 albino guinea pigs, anesthetized with Nembutal (25 mg/kg, i.p.), fentanyl (0.2 mg/kg, i.m.) and droperidol (10 mg/kg, i.m.). In some experiments, dexamethasone (1.5 mg/kg, i.p.), atropine (0.05 mg/kg, s.c.) and xylocaine (s.c.) were used as needed. Pinnae were removed bilaterally and cochlear nuclei visualized after cerebellar aspiration through a posterior craniotomy. After the craniotomy, the animal was further anesthetized with urethane (1.0 g/kg, i.p.), and maintained with occasional dose of additional fentanyl and droperidol to ensure absence of toe pinch reflex.

2.2.2 Electric Stimulus Electric stimuli were delivered through a single stainless steel wire (400 μm) electrode with an impedance of 10–40 kΩ. The return electrode
was inserted in a neck muscle. The electrode penetrated the CN from its dorsal surface. Through this electrode, monophasic electric pulses delivered by a current source (A-M Systems model 2200).

The limitation for shock level was twitching or appearance of large irregular waves. Occasionally, abnormal respiration was seen with stimulation. Any data collected with such problems, or EABR that was unstable or excessively noisy was excluded from the analysis.

Typically, 50 μs-long monophasic pulses were presented, in the current range of 12 to 250 μA. This was empirically determined using a guideline that the EABR amplitude should not disproportionately exceed the general range of the ABR wave amplitudes (10–20 μV) obtained with the same preparation and measurement system but using acoustic clicks or chirp stimuli.

2.2.3 Acoustic Stimulus All experiments were performed in a booth shielded acoustically and electrically. EABRs were recorded in silence, but in one case (Figure 6), free field acoustic noise of about 95 dB SPL was generated using two loudspeakers.

2.2.4 EABR recording To record the EABR, a recording electrode was placed at epidural vertex, and the reference electrode was inserted in facial muscles around the ipsilateral ear, where the skin was removed with the pinna. The signal was AC coupled and amplified by 80 dB using an Ithaco amplifier model 1201, with minimal filtration. The signal was sampled at 80 kHz and stored in a computer. After the experiment, the stimulus artifact was suppressed by subtracting the best-fitting exponential function modeling the artifact for each presentation of stimulus pulse, and then averaged over 20 to 500 trials. The EABR was recorded with stimulus pulse rates from 1.0 to 17.8 Hz, and waveforms were pooled and averaged across the stimulus rates. Stimulus pulse was always presented at time 0 in traces, and the following 75 μs of the data is blanked, where residual artifact can be large.

For this study, analyses included only those waveforms from stimulation sites wherein 60 μA cathodic stimulation evoked more than one wave. Waveforms with only one apparent wave were excluded since there is no reliable way to test whether the apparent wave is residual artifact or legitimate wave of neural origin. For analyses that used a standardized stimulus level of 30 or 60 μA, such responses were interpolated from two adjacent stimulus levels when data was unavailable at the exact stimulus level.

2.2.5 Histology After EABR recording, if deemed particularly valuable, the stimulation site was marked by passing DC current of 100 μA for 20–30 seconds (a total of 2–3 mC), whereby ferric ion is deposited in situ.

Most animals were sacrificed by transcardial perfusion using a fixative containing 10 g potassium hexacyanoferrate (II) trihydrate, 10 g glutaraldehyde, 30 g formaldehyde in one liter of 0.01M phosphate buffered saline (pH=7.4). In several cases, the animal was sacrificed by overdose of urethane and the head was immersion fixed, using a modified fixative containing 25 g glutaraldehyde and 25 g formaldehyde
Hexacyanoferrate (II) reacts with the ferric ion deposited by the stainless steel stimulating electrode and the DC current injection to form Prussian blue pigment at the site of electrode placement.

The fixed brainstem was immersed in 30% sucrose solution overnight and then sectioned in the transverse plane on a freezing microtome in 80μm thickness. Sections were counterstained with neutral red.

The site of stimulation was identified from the Prussian blue staining (Figure 1b), electrode tracks without marks (14 sites), and sketches of the stimulating electrode entry into the CN made during experiments (all 15 sites). Staining typically extended through multiple sections, and we sought agreement between the ventral-most extent of the electrode track and most intense or extensive blue staining as the best representation of the actual stimulation site.

The CN subdivisions are identified using the histological criteria of Hackney et al. (1990) and compared to the sites of the stimulation. A relative anterior-posterior dimension (A-P dimension or scale) was calculated from the number of sections with reference to the section containing the dorsalmost extension of auditory nerve root (ANR, A-P scale of 0), and normalized by the size of the particular AVCN or PVCN size. The most rostral section containing AVCN is 100, and the most caudal section containing PVCN, −100. AVCN typically contained 25 sections of 80μm (sample median); thus the A-P scale of 100 typically represents 2.0 mm anterior to the dorsalmost extension of the ANR. Similarly, PVCN contained 14 sections, thus −100 typically represents 1.12 mm posterior.

2.3 Results

EABR waveforms were recorded from stimulation in 12 cochlear nuclei in 12 animals (Table 1). From them, we selected stimulation sites within CN or 8th cranial nerve. Analysis included stimulation sites where waveforms elicited by cathodic pulses of between 30 and 60μA comprised more than one wave and could therefore be convincingly of neural origin rather than residual artifact.

2.3.1 Stimulation in CN away from 8th nerve

A typical example of an EABR from a CN site is shown in Figure 1(a). The EABR had three major vertex-positive waves. The first wave had a peak appearing at 0.40–0.47 ms, the second wave 0.93–0.96 ms, and the third 1.59–1.62 ms (the range for three traces in Figure 1a). The first wave was usually within the stimulus artifact and became discernible only after artifact suppression. All three EABR waves increased with increasing shock level. At 62μA stimulus, the amplitude of the first wave was 41μV (peak to pre-stimulus baseline) and the later waves were at least 10μV.

These EABRs on Figure 1(a) were evoked by a stimulating electrode located at the site shown in Figure 1(b). The site was in the posterior part of the AVCN but it was rostral to the auditory nerve root. The section containing the dorsalmost extension of the ANR (A-P scale of 0) was 400μm caudal to this section. In the dorso-ventral
Fig. 1. (a) EABR waveforms in response to cathodic pulses of three different current levels (50 μs duration, presented at the beginning of the blanked period). The stimulation site was AVCN (Figure 2, case 98–13). Latencies, 16 μA: 0.47, 0.97, 1.62 ms. 31 μA: 0.41, 0.96, 1.59 ms. 62 μA: 0.40, 0.93, 1.59 ms. Amplitudes of the first waves, in μV re pre-stimulus baseline: 8.3 at 16 μA, 19.3 at 31 μA, 40.6 at 62 μA. (b) A transverse section of lower pons with Prussian blue staining in posterior AVCN indicating the location of the stimulating electrode used to evoke the response waveform. This section is 5 sections anterior to the dorsalmost extension of the ANR, and this AVCN spanned a total of 16 sections. Thus the A-P scale for this site is (5/16)100 ≈ 31.

dimension, the site was in the ventral half of AVCN but no closer than 500 μm from the auditory and vestibular nerve roots.

EABRs were recorded from a total of 7 histologically confirmed sites that were in CN (Figure 2) and located more that 300 μm away from the 8th nerve. Along the anterior-posterior dimension, the amplitudes and low frequency components of the EABR waveforms changed, but general features of the waves did not vary significantly (Figure 2). Typically, the first wave appeared about 0.3 ms after the stimulus, the second wave 0.8–1.2 ms, and the third wave 1.6–2.2 ms. There may be a suggestion of another wave at 0.6 ms (Figure 2, cases 107–39 and 112-12). Some waveforms contained another wave at approximately 3 ms (Case 97–14).

2.3.2 Stimulation in ANR area Stimulation in the ANR area produced EABR waveforms (Figure 3) with somewhat more complex morphology than CN cellular area stimulation (Figure 2). They comprise 3 or more major waves, but there are more suggestions of minor waves and negative waves at 0.75 ms.
2.3.3 **Stimulation at all CN sites** The amplitude of the EABR first wave was larger in AVCN than in PVCN (Figure 4, 5). The largest EABR response in Figure 2 was from case 98–13, the AVCN stimulus site shown in Figure 1 (b), at an A-P scale of 31. The smallest was from case 123–95, A-P scale of −36 (PVCN).

2.3.4 **Effects of acoustic noise and stimulus polarity** In order to test whether the generators of these waves are indeed within the auditory system and not from nearby structures, in one case, EABRs were recorded with and without loud acoustic noise. The EABR waves were smaller with noise (Figure 6). The amplitudes decreased in noise presumably because a fraction of the neural generators of the EABR were refractory and non-responsive to the electric stimulus while responding to noise vigorously.

Cathodic stimulation was typically between twice and four times more effective than anodic stimulation, i.e., anodic stimulation required higher level to produce...
Fig. 3. EABR waveforms recorded from stimulation in 5 sites in ANR area, arranged from most posterior (top, at A-P scale of -30) to most anterior (bottom, A-P scale of 26). All traces were obtained with 60 μA cathodic stimulus. One site (case 102-46) did not have histology, but its location estimated from visual observation. Waveforms here are less stereotyped than in Figure 2.

A response amplitude equal to that for cathodic stimulation. Figures 7 and 8 show waveforms from two different preparations, where waves grow with stimulus level, and cathodic pulses evoked larger responses in the entire range of stimulus levels. Highest stimulus level usable in most cases was 125 to 250 μA.

The polarity of the first wave is positive with cathodic stimulation, but negative with anodic stimulation. The waveform morphology is more complex with anodic stimulation, and the complexity increases with stimulus level. As the anodic stimulus level is increased, a positive wave may appear amidst the initial negative wave (pointed by an arrow in Figures 8, 250 μA anodic stimulation). The increased waveform complexity suggests that anodic stimulation may be recruiting additional cellular generators, especially at higher levels.

2.3.5 Stimulation in or near vestibular structures  Stimulation in or near the vestibular nerve or Scarpa’s ganglion (5 sites) produced another type of waveform (Figure 9) at the lowest stimulus levels used (12–22 μA). Stimulation within vestibular structures (VNR: 95–5 and 108–67; Scarpa’s ganglion: 96–59) produced waveforms comprising 2 major waves with similar latencies. On the other hand, stimulation in AVCN-p by VNR (108–93), at 12 μA, produced EABR waveform very similar to those seen from other AVCN sites distant from vestibular structures (Figure 2), but the waveform morphol-
Fig. 4. Amplitudes of the first wave in EABR from all 11 histologically verified sites versus A-P scale. The amplitude was measured from pre-shock baseline to peak. The amplitude of EABR obtained is large in AVCN-p and it is small in PVCN-p. Cathodic 60 µA stimulus was used.

Fig. 5. Atlas section illustrating stimulation sites within CN (including nerve root area). The numbers below are the A-P scale. Sections are arranged in increasing A-P scale, from posterior (A-P scale of −50) to anterior (A-P scale of 50). Symbols represent the amplitude ranges for the EABR first wave at 60 µA cathodic stimulus.
<table>
<thead>
<tr>
<th>Case ID</th>
<th>A-P scale</th>
<th>Location</th>
<th>1st wave amp. (µV)</th>
<th>Figure(s)</th>
<th>Remarks</th>
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<td>-50</td>
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<td>7</td>
<td>2, 4, 5</td>
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<tr>
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<td>6</td>
<td>2, 4, 5, 8</td>
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<td>-30</td>
<td>PVCN by ANR</td>
<td>6</td>
<td>3, 4, 5</td>
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<tr>
<td>99-9</td>
<td>8</td>
<td>ANR</td>
<td>24</td>
<td>3, 4, 5</td>
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<tr>
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<td>ANR</td>
<td>30</td>
<td>3</td>
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<tr>
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<td>18</td>
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<td>23</td>
<td>2, 4, 5</td>
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<td>22</td>
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<td>9</td>
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Table 1. List of cases included for analysis of the present study. The first wave amplitude was measured with 60 µA cathodic stimulus, and peak voltage was measured against pre-stimulus baseline. Case 97-14: EABR was recorded in silence and in noise. The first wave amplitude was 17 µV in noise. Case 95-5: the waveform changed so considerably between 16 and 31 µA stimuli that measurement at 62 µA is not interpretable. Cases 123-95 and 112-12: anodic stimulation traces are shown in Figures 7 and 8.

Fig. 6. EABR waveforms recorded from stimulation in AVCN (case 97-14, A-P scale of 32). Solid trace was recorded in quiet, and dashed trace in acoustic noise (approx. 95 dB SPL). Both traces were obtained with 60 µA cathodic pulses. The EABR waves were smaller in presence of acoustic noise, indicating that the EABR neural generators are acoustically responsive.
Fig. 7. EABR waveforms for increasing stimulus levels with stimulation in AVCN, A-P scale of 57 (Case 112–12).

ogy became more complex, incorporating the two-wave morphology, as the stimulus level increased (data not shown). Stimulation in AVCN adjacent to Scarpa’s ganglion (96–24), waveform was already complex at the lowest stimulus level used (16 µA) but the waveform morphology of direct vestibular stimulation became more pronounced at 62 µA stimulus (data not shown).

Level series waveforms from two sites in VNR (Figure 10, 108–67) and Scarpa’s ganglion (Figure 11, 96–59) show increased complexity in the waveform as the stimulus level increased, suggesting gradual recruitment of distant generators. When the electrode was in VNR (Figure 10) or Scarpa’s ganglion (Figure 11), at the lowest stimulus levels, the gross potential waveforms shared the same morphology, comprising two waves at the same latencies. However, at higher stimulus levels, the waveform became more complex and generally incorporated features of EABR from AVCN or auditory nerve stimulation.

2.4 Discussion

2.4.1 Consistency of EABR waveforms Our results demonstrate that focal stimulation of CN resulted in EABR waveforms with three major peaks. The waveform was generally similar in all parts of the ventral CN, suggesting one major cell type in CN is excited by electric stimulation and is the major contributor to the EABR. This find-
Cathodic Stimulation Anodic Stimulation

Fig. 8. EABR waveforms for increasing stimulus levels with stimulation in PVCN, A-P scale of −36 (Case 123–95). Arrow at bottom right indicates a small positive peak within a large negative wave.

...ing suggests that varied morphologies of waveforms seen in previous studies of EABR might be a result of non-specific stimulation. Within the present study, non-specific stimulation was seen in the form of complex waveforms, and we were able to clearly recognize this aspect of non-specific stimulation owing to the concurrent effort to obtain clean and uncontaminated waveforms.

Cochlear nucleus is surrounded by excitable neurons, which may contaminate EABR when stimulation is not specific to CN neurons. This study (Figures 8, 10 and 11) demonstrated that vestibular structures can generate distinct waves in gross potential and lend mixed morphologies to EABR waveforms (Figure 9, cases 96–24, 108–93; Figure 11 at 62 μA). One potential source of variability, then, in previously reported EABR waveforms, is the contribution of non-CN neurons from non-specific stimulation.

In this study, low stimulus levels were primarily used in order to keep the stimulation as site-specific as possible. Structures with large axons, such as vestibular nerve or ganglion, produced clean and reliable responses with 12 to 22 μA cathodic stimuli (Figure 9), although 30 to 63 μA stimuli improved the signal-to-noise ratio from CN and ANR stimulation without altering the waveform morphology (Figures 1 (a), 7 and 8). High stimulus levels, which may recruit additional neural generators, may account for waveform diversity in previous studies.

The stimulus level of 30 to 60 μA used in this study may excite axons as far as 200 to 400 μm for the thickest axons included in study by Ranck (1975). For thinner
Fig. 9. Gross potential waveforms from stimulation in 5 sites in or near vestibular structures, arranged from most posterior (top, at A-P scale of 21) to most anterior (bottom, A-P scale of 33). Case 108-67 was in VNR near ANR, 96-24 AVCN near VNR, 108-93 in AVCN near VNR, 95-5 Scarpa’s ganglion near AN and AVCN, and 96-59 Scarpa’s ganglion near AVCN. All traces were obtained with 16 µA cathodic stimulus, except for 12 µA (case 108–93) and 22 µA (case 108–67).

axons to be excited, they must be within a smaller radius. Of all the studies wherein the CN is stimulated, ours is the only one to verify the site of stimulation by histology, and also the only one to obtain waveforms of consistent morphology, across animals, from stimulus parameters designed to contain radius of excitation well within the CN.

2.4.2 Generators of the first wave of EABR from stimulation of CN cellular areas The first wave in the EABR appeared at a latency of 300 to 400 µs. This wave is presumably generated by direct stimulation of CN neurons, auditory nerve, vestibular nerve, or combination thereof, as such a short latency precludes the action potentials from postsynaptic neurons.

Effective cellular generators of EABR have thick axons and large population size because the former renders cell more excitable, and both factors contribute to generation of a large far field potential. Numerous cells are required for their gross potential to be sizable. Candidates for the generators are, in decreasing order of axon diameter thus excitability, globular bushy cells (axon diameters around 10 µm in cats), vestibular nerve (10 µm in cats; Walsh et al. 1972), spherical bushy cells (4 µm in cats) and AN (2–3 µm in guinea pigs; Brown 1987). Among the AVCN cells, globular bushy cells are the most likely candidate of generator, analogous to the generator of the P2 wave in ABR, but spherical cells and some auditory nerve may also be involved. The globular bushy cells are located in PVCN-a and AVCN-p (Hackney et al. 1990), which is
Fig. 10. EABR waveforms from a site in VNR, at A-P scale of 21. The responses at 22 and 44 μA (top two) contain two waves, but the waveform becomes more complex with increasing stimulus level, presumably because auditory nerve is recruited (case 108-67).

Fig. 11. EABR waveforms from a site in Scarpa's ganglion, at A-P scale of 33 (equivalent). The response at 16 and 31 μA (top two) contains two waves, but the waveform becomes more complex with increasing stimulus level, presumably because AVCN neurons and auditory nerve are recruited (case 96-59).
roughly in agreement with the distribution shown in Figures 4 and 5.

The second wave at 0.8–1.2 ms and the third wave at 1.6–2.2 ms of CN stimulation (Figure 2) are probably generated by the downstream targets of bushy cells, such as MNTB and VNLL, as these waves can be identified even at very low stimulus levels (10–30 μA) where other cell types are unlikely to be excited.

Stimulation of multipolar cells at low current levels (16–63 μA) is unlikely. As a part of a separate research, PVCN-a was focally stimulated with the same stimulation system while monitoring DPOAE in the ear ipsilateral to the CN. When PVCN neurons were stimulated, medial olivocochlear (MOC) reflex was activated and the DPOAE level changed. In order to see such effect reliably, stimulus level of 125 μA or larger was necessary, presented at 200 or 400 Hz. Most numerous cells in PVCN-a are multipolar cells, a subset of which is thought to be MOC interneurons.

The amplitude of the first wave was reduced in presence of acoustic noise (Figure 6). This suggests that the EABR was generated by neurons that are driven by sound. However, since the discharge pattern with acoustic noise is asynchronous to the pulse timing, and therefore only a small fraction of the EABR generator would be refractory, the reduction in EABR amplitude is small. Similarly small reductions were observed in compound antidromic action potential evoked by electric stimulation of the auditory nerve (Brown 1994).

2.4.3 Generators of the first wave of EABR from ANR stimulation

The negative first wave seen in response to anodic stimulation (Figures 7 and 8) is presumably generated by the AN. Antidromic firing of AN is expected to cause such a negative EABR wave because of the direction of propagation away from the vertex electrode and toward the reference electrode placed in the ipsilateral ear (Nunez 1981). Such negative wave is repeatedly observed with anodic stimulation in the CN but not cathodic stimulation. In general, cathodic stimulation is more effective in stimulating cell bodies and axons (Ranck 1975).

Auditory nerve central to the bifurcation point is significantly thinner than at more distal points. Since thicker axons are more readily excitable (Ranck 1975), the most effective place to excite is a node of Ranvier on the main fiber. With anodic stimulation in the CN, current pushes into the AN in the CN and it depolarizes the axonal membrane as the current leaves the AN, where action potentials may be discharged. Thus induced action potential may propagate both directions, into the CN and also toward cochlea. The latter antidromic portion appears in the EABR as a negative wave, since it travels toward the reference electrode. The orthodromic portion is unlikely to contribute to EABR, as ascending and descending fibers are both thinner (1.75 and 1.17 μm, respectively) than the main fiber (2.14 μm) on average (Brown 1987), but their targets may.

EABR waveforms with positive first wave, such as those from cathodic stimulations, indicate that the response from the CN neurons overpowered the potential wave from AN, and they do not guarantee absence of AN stimulation, especially at high stimulus levels. This probably acts as a source of negative bias for the amplitude
of the wave generated by the CN neurons. Similarly, EABR waveforms with negative first wave means that the response from the AN overpowered the potential wave from CN neurons. Indeed, waveforms in Figures 7 and 8 (arrow) for anodic 250 μA stimulation indicate signs of positive waves within the first negative wave.

### 2.4.4 Generators of the first wave from stimulation of vestibular structures

Vestibular nerve is more excitable with cathodic than anodic stimulation, like most other neurons. Unlike AN, antidromic action potential of the vestibular nerve does not appear to contribute much to the gross potential, as seen in the absence of negative waves in Figure 9. Waveforms obtained from cases 108–67, 95–5 and 96–59 (Figure 9) are similar to gross potentials evoked by vestibular nerve stimulation (Berryhill and Javel 2001; van den Honert and Stypulkowski 1986).

In Figures 10 and 11, waveforms were compared with a series of stimulus levels. At the lowest stimulus level, only one generator was presumably excited, and another generator was excited at higher stimulus levels.

### 2.5 Acknowledgements

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EABR of cochlear nucleus


EABR of cochlear nucleus


Chapter 3

Site-specific DPOAE effects from focal electric stimulation of cochlear nucleus

Keywords: MOC, olivocochlear reflex interneurons, AVCN shell, PVCN, auditory brainstem, distortion product

Abstract The medial olivocochlear (MOC) reflex is a sound-evoked reflex that proceeds through the cochlear nucleus (CN) and has its action in the cochlea. To identify the location of reflex interneurons in the CN, we used focal electrical stimulation of different subdivisions while monitoring acoustic levels of distortion product otoacoustic emissions (DPOAEs) in the ear canal. DPOAE levels changed when the stimulating electrode was located in or immediately adjacent to the posteroventral CN (PVCN), or the anteroventral CN (AVCN) shell, but not when the electrode was in the dorsal CN or the core of AVCN. This indicates that both PVCN and AVCN shell furnish excitatory inputs to MOC neurons. Furthermore, surgically severing the intermediate acoustic stria did not abolish the effect of PVCN stimulation, and stimulating sites near the ventral acoustic stria (VAS) caused DPOAE changes, indicating that the reflex interneurons send axons through the VAS.

3.1 Introduction

The CN is the gateway to the central auditory system. Its inputs from the auditory nerve (AN) inputs drive multiple cell types in different subdivisions (anteroventral CN, AVCN; posteroventral CN, PVCN; dorsal CN, DCN), with each cell type in turn projecting centrally to different targets (Rhode and Greenberg 1992). Thus the CN is the origin of the parallel pathways in the central auditory system. One or more of these neurons must be involved in the medial olivocochlear (MOC) reflex. MOC neurons originate in the superior olivary complex, and convey sound-evoked information to the outer hair cells (OHC) in the cochlea (Figure 1). The intermediate limbs of the pathways involve the cochlear nucleus (Robertson and Winter 1988; Thompson and
DPOAE effects from electric stimulation of cochlear nucleus

Fig. 1. Schematic showing the methods of this study and the neural pathways involved. A stimulating electrode is placed in the cochlear nucleus (CN) on one side, defined as the ipsilateral side. If the electrode is located near the MOC reflex neurons (purple color), they become activated by the electric stimulation and convey their messages to the MOC neurons located predominantly on the opposite side of the brainstem (most projections from the cochlear nucleus decussate). MOC neurons consist of two groups, ipsi neurons, which preferentially respond to sound in the ipsilateral cochlea, and contra neurons, which preferentially respond to sound in the ipsilateral cochlea. The ipsi neurons re-decussate and project to the ipsilateral cochlea, altering the DPOAE measured in that ear canal. The contra neurons project to the contralateral cochlea, altering the DPOAE in that ear canal.

Thompson 1991; Ye et al. 2000), but which population of cochlear nucleus neurons functions as the reflex interneuron (de Venecia et al. 2005; Thompson and Thompson 1991; Ye et al. 2000) is not understood. The present study explores which neurons of the cochlear nucleus are interneurons, by using focal electric stimulation applied to subdivisions of the cochlear nucleus (Figure 1).

Direct projections from the ventral cochlear nucleus (VCN) to the MOC neurons have been observed anatomically (Robertson and Winter 1988; Thompson and Thompson 1991; Ye et al. 2000; also see Warr 1969) using double labeling techniques, where tracers were injected in cochleae and cochlear nuclei, observing CN axon terminals contacting the MOC neurons. However, these studies suggested different subdivisions of the VCN as the location of MOC interneurons. Thompson and Thompson (1991) injected tracers in PVCN and cochlea and observed labelled CN neuronal terminals contacting the labeled MOC neurons. On the other hand, Ye et al. (2000) injected tracers to the marginal shell of the cat AVCN and cochleae, and observed retrogradely labeled CN neuronal terminals contacting the labelled MOC neurons. An additional question as to whether each MOC input is excitatory and which is sufficient to activate MOC reflex independent of the state of the other pathway.

One previous physiological study used selective lesions made by the neurotoxin kainic acid (de Venecia et al. 2005). This study showed that a lesion in PVCN was suffi-
cient to permanently eliminate acoustically evoked MOC effect as assayed by DPOAE, suggesting that PVCN is the location of sound-evoked MOC interneurons. However, whether activities of these neurons are sufficient to activate the MOC reflex could not be addressed in that study, but it can be answered with the methods used in this study.

To identify the location of such excitatory inputs to MOC neurons, we used focal electric pulses to stimulate a subset of the CN parallel pathways (Figure 1). Our metric for activation of the MOC reflex takes advantage of projection to the OHC via changes in their distortion product otoacoustic emissions (DPOAEs; Mountain 1980, Siegel and Kim 1982, Deeter et al. 2009), wherein a change in DPOAE level from the baseline level may be construed as an indication of MOC reflex being activated. DPOAEs were measured on both the ipsilateral and contralateral sides (Fig. 1), because CN interneurons project to MOC neurons that in turn project back to the ipsilateral cochlea (MOC Ipsi neurons) and that project to the contralateral cochlea (MOC Contra neurons). Thus the general approach of this research is to identify the location within cochlear nucleus wherein electric stimulation leads to a change in DPOAE level, an indication of MOC reflex activation.

3.2 Methods

3.2.1 Animal preparation All experimental procedures on animals were in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals, and were performed under approved protocols at the Massachusetts Eye and Ear Infirmary. Experiments were performed with 27 albino guinea pigs, anesthetized with Nembutal (25 mg/kg, i.p.), fentanyl (0.2 mg/kg, i.m.) and droperidol (10 mg/kg, i.m.). In some experiments, dexamethasone (1.5 mg/kg, i.p.), atropine (0.05 mg/kg, s.c.) and xylocaine (s.c.) were used as needed. Pinnae were removed bilaterally and cochlear nuclei were visualized after cerebellar aspiration through a posterior craniotomy. After the craniotomy, the animal was further anesthetized with urethane (1.0 g/kg, i.p.), and maintained with occasional dose of additional fentanyl and droperidol to ensure absence of toe pinch reflex.

In 4 animals (7 sites total), D-tubocurarine (1.2 mg/kg i.m.) was used to paralyze the animal. In such cases, the animal was ventilated with an artificial respirator (4 ml/stroke, 60 strokes/s).

Electric Stimulus The stimuli are delivered through a paired stainless steel wire electrode with an impedance 10-40 kΩ. The electrode penetrated the CN from its dorsal surface. Through this electrode, monophasic electric pulses are delivered via a transformer. Typically, 200 μs-long monophasic pulses were presented at 200 Hz, with the voltage of 1 to 20 V (typical current of about 0.2-1.5 mA). Each run of stimulus contained such a pulse train lasting from 10 to 120 s, with at least a few seconds of leading and trailing blank period where no stimulus pulse was presented.

The most significant limitation for shock level was twitching. Occasionally, abnormal respiration or change in primary tone levels were seen with stimulation. Any
data collected with such problems, or unstable or noisy DPOAE baseline was excluded from the analysis. Stimulus level was also limited so that the primary tone levels stayed within 1.0 dB of the baseline value to exclude potential activation of middle ear muscle reflex. A small change in primary tone levels is expected since MOC reflex can alter the acoustic impedance of the cochlea. Anterior AVCN was particularly susceptible to twitching and abnormal respiration problems.

The range of stimulating VAS and OCB is estimated to be about 500 to 750 μm. The strongest stimulus current used was 1 to 1.5 mA based on the electrode impedance, but most data were obtained with 200 μA to 1 mA current level. Information on the range of stimulation is available for monopolar electrodes (Ranck 1975), wherein the range of stimulation was about 500 to 1000 μm for thick myelinated axons and cell bodies using 200 μs monophasic pulse of 200 μA delivered by a monopolar electrode. In an additional experiment conducted using a current controlled stimulus delivered by a monopolar electrode, 3 to 5 times less current was required than with a bipolar electrode for a comparable range of stimulation. Thus, we used 750 μm distance as the criterion for proximity to VAS and OCB. MOC axonal diameter in guinea pigs is 1.2–1.4 μm (Brown 1989), and therefore MOC neurons are expected to require stronger stimulus than the thick axon data in Ranck (1975).

3.2.2 Assay for MOC activation Distortion product otoacoustic emission (DPOAE) was produced by two primary tones, f<sub>2</sub> selected from 8 to 12 kHz, and f<sub>1</sub>, 0.83 f<sub>2</sub>. The f<sub>2</sub> primary tone is presented 10 dB higher than f<sub>1</sub>, i.e., L<sub>2</sub> = L<sub>1</sub> + 10 (dB) where L<sub>1</sub> was typically selected from 40–60 dB SPL, and DPOAE at 2f<sub>1</sub> − f<sub>2</sub> was recorded; the acoustic pressures of the primary tones were also recorded in the external ear. The primary tones were presented and DPOAEs measured with an Etymotic ER−10c transducer. The microphone signal was amplified and sampled at 250kHz and quantized in 16 bits. Twelve non-overlapping adjacent time series signals were averaged before the spectral powers were evaluated at f<sub>1</sub>, f<sub>2</sub> and 2f<sub>1</sub> − f<sub>2</sub>. Two power spectra were averaged for final measurement value, providing approximately one DPOAE measurement per second.

Cochlear nucleus sites were focally stimulated while recording distortion product otoacoustic emissions (DPOAEs), where the onset and offset timing of the shock train presentation was synchronized to the signal averaging buffers (Figure 2a).

In some cases, DPOAE was measured in the ipsilateral and contralateral ears at same f<sub>1</sub> and f<sub>2</sub>. Primary levels were adjusted to obtain a comparable DPOAE baseline. If unspecified, DPOAE measurement refers to one measured in the ear ipsilateral to the CN being stimulated. DPOAE effect is defined as the maximum change in DPOAE, while the shock is presented, from the pre-shock baseline.

3.2.3 Surgical cuts The dorsal and intermediate acoustic striae were surgically cut with a small knife in 5 cases. The location of the cut is dorsal to restiform body, near the medial end of the dorsal cochlear nucleus at a depth of approximately 1.5 mm, which is enough to cut these two acoustic striae but too shallow to affect the MOC fibers in brainstem. The effect of lesion is typically assayed at the contralateral ear
to avoid the possible risk of MOC fiber damage, but whenever ipsilateral ear data is used, absence of MOC fiber damage is confirmed histologically.

### 3.2.4 Histology

Selected sites of stimulation were marked by passing DC current of 100 μA for 20–30 seconds (a total of 2–3 mC), after all necessary data were obtained, whereby ferric ion is deposited in situ, for later reaction with hexacyanoferrate (II) to form the Prussian blue pigment. The animal was then fixed by transcardial perfusion using a fixative containing 10 g potassium hexacyanoferrate (II) trihydrate, 10 g glutaraldehyde, 30 g formaldehyde in one liter of 0.01M phosphate buffered saline (pH=7.4). In several cases, the animal was sacrificed by overdose of urethane and the head was immersion fixed, using a modified fixative containing 25 g glutaraldehyde and 25 g formaldehyde (pH=7.4). Hexacyanoferrate (II) reacts with the ferric ion deposited by the stainless steel stimulating electrode and the DC current injection to form Prussian blue pigment at the site of electrode placement (Figure 2b).

The fixed brainstem was sectioned in the transverse plane on freezing microtome in 80 μm thickness, and the sections were counterstained with neutral red. Some specimens were also stained for acetylcholine esterase. The site of stimulation was identified with Prussian blue staining (34 sites), electrode track without marks (27 sites), and sketches made during experiments (all sites). The CN subdivisions were identified using the histological criteria of Hackney et al. (1990) and compared to the site of the stimulating electrode as determined by the electrode track and blue pigment. A relative anterior-posterior dimension (A-P dimension or scale) was calculated from the number of sections with reference to the section containing the dorsalmost extent of auditory nerve root (ANR), and normalized by the size of the particular AVCN or PVCN size. An A-P scale of 0 is assigned to the transverse section containing the dorsalmost extension of the of ANR. The most rostral section containing AVCN is 100, and the most caudal section containing PVCN, −100. AVCN typically contained 25 sections of 80 μm (sample median), thus the A-P scale of 100 typically represents 2.0 mm anterior to the peak of ANR. Similarly, PVCN contained 14 sections, thus −100 typically represents 1.12 mm posterior to the peak of ANR. Several sections posterior to −100 contained DCN only. This dimension is used rather than absolute distance to absorb the size variation of individual CN. Sites that were within 750 μm of olivocochlear bundle or ventral acoustic stria were also noted for the approximate distance from these structures.

### 3.3 Results

#### 3.3.1 DPOAE Effect

The DPOAE level changed significantly when electric shocks were delivered to some CN sites. An example is shown in Figure 2 (a). The maximum absolute change in DPOAE level during the shock presentation period, which typically occurred following the shock onset, is hereafter referred to as the DPOAE effect and used as the metric of the MOC activation. When the DPOAE level changed, it typically decreased, but in some sites, it increased from the pre-stimulus baseline level. Only data with little or no change in the primary tone levels (less than 1.0 dB) were in-
Fig. 2. (a) DPOAE and primary tone levels time courses for electric stimulation of the cochlear nucleus, where the measurements were made in the ear ipsilateral to the CN being stimulated. The shock train started immediately after the measurement at time $-1$ s, and ended after 24 s. Arrows indicate DPOAE effect, defined as the maximum change in DPOAE relative to the pre-shock baseline. The DPOAE effect in this case is 11.3 dB (DPOAE level changed from 8.2 dB to $-3.1$ dB SPL). The actual pre-shock baseline of primary tone levels was $L_1$ 44.9 dB SPL, and $L_2$ 54.9 dB SPL, shown with constant offsets. (b) A transverse section of lower pons with Prussian blue mark indicating the location of the stimulating electrode in dorsomedial PVCN. The DPOAE data in Figure 1 was obtained by stimulating at this site. This section is 8 sections posterior to the dorsalmost extent of the ANR, and this PVCN was contained in 14 sections. Thus the A-P scale for this site is $(8/14)100 \approx 57$.

cluded for analysis to avoid issue of potentially stimulating middle ear muscle reflex (See Figure 7 for summary of all data points). In most experiments, the animals were not paralyzed while measuring DPOAE effects. However, in 4 cases, animals were paralyzed after initial unparalyzed measurements; DPOAE effects remained within normal repeatability range.

3.3.2 Effects from PVCN stimulation The site of stimulation was confirmed through postexperimental histology. Figure 2 (b) shows a section showing the Prussian blue reaction at the electrode site in dorsomedial PVCN, the stimulation site used in Figure 2 (a).

Among the sites within the CN core, as shown in Figure 3, the DPOAE effect was large in PVCN, particularly in the posterior half of PVCN (A-P scale between $-100$ and 0, particularly between $-80$ and $-50$). The majority of the sites that produced DPOAE effect greater than 2.5 dB were PVCN sites.

Most of the effective PVCN stimulation sites were located dorsomedially, although there are few data points from ventral PVCN. Each data point from Figures 3
Fig. 3. DPOAE effect against anterior-posterior dimension (A-P scale) of stimulation site within CN core, where $-100$ defined to be the section containing the posterior end of PVCN, 0 the dorsalmost extent of ANR, 100 the anterior end of AVCN. Typically, $-100$ is 1.12 mm posterior to ANR, whereas $+100$ is 2.0 mm anterior to ANR. Each filled circle indicates DPOAE effect from a VCN site measured in the ear ipsilateral to the stimulated CN, and unfilled circle, the contralateral ear. Each filled triangle indicates a DPOAE effect from a DCN site measured in the ipsilateral ear, and unfilled triangle, the contralateral ear. This figure contains stimulation sites within CN core and excludes sites in AVCN shell, or sites within 750 μm of VAS or OCB (Cf. Figure 4).
3.3.3 Effects from AVCN shell stimulation Besides PVCN, large DPOAE effects were observed from stimulation in some sites in or immediately adjacent to AVCN shell (Figure 4, panel A). Among 9 sites in or immediately adjacent to AVCN shell, 5 sites produced DPOAE effects larger than 2.5 dB. In one of the 5 sites, DPOAE was measured bilaterally, and effects were larger than 2.5 dB in both ears. In one AVCN shell site, DPOAE effect measured in the ear ipsilateral to the CN was 6.2 dB, which decreased to 2.7 dB after a cut along the 4th ventricle. At this site, DPOAE effect in the contralateral ear was 3.8 dB before the cut.

3.3.4 Lack of effects from stimulation of AVCN core and DCN In contrast to PVCN and AVCN shell, most stimulation sites in AVCN core and DCN did not produce large DPOAE effects. Among limited data available from AVCN core that were more than 500 µm from VAS, OCB, ANR or PVCN, 0 out of 4 sites produced DPOAE effects larger than 2.5 dB (Figure 3).

DCN stimulation was ineffective and did not produce large DPOAE effects. No stimulation site in DCN produced DPOAE changes greater than 2.5 dB, except for one site that was within 150 µm of effective PVCN sites (shown in -75 section in Figure
Fig. 5. DPOAE time course measured in the ear contralateral to the stimulated PVCN, before (broken line) and after (solid line) cutting the IAS and DAS next to the PVCN being stimulated. The shock train started immediately after the measurement point at time $-i$, and ended after $i$. The pre-shock baseline DPOAE levels were 10.28 dB SPL (before cut) and 10.79 dB SPL (after cut), but the two traces are aligned with a constant offset for comparison. The DPOAE effects were 3.7 dB (before) and 3.9 dB (after), indicating that cutting the IAS and DAS of the CN being stimulated did not have significant effect on the DPOAE. The maximum change in the primary tone levels was 0.1 dB for both $f_1$ and $f_2$, before and after the cut.

3.3.5 IAS/DAS cuts and stimulation of VAS In some experiments, DPOAE effects were measured with PVCN stimulation, before and after the intermediate acoustic stria (IAS) and dorsal acoustic stria (DAS) were surgically severed medial to CN. The DPOAE effect was typically unaffected and never abolished completely. Figure 5 shows DPOAE traces from a PVCN stimulation site, showing little change after the cut. Postexperimental histology indicated that the cut was deep enough to interrupt both IAS and DAS.

Large DPOAE effects were observed from stimulation in sites in close proximity to VAS, as shown in Figure 4, panel B, which shows the DPOAE effects of sites within 750 μm of VAS, plotted against the A-P scale.

3.3.6 Summary of results Overall, stimulation in PVCN and AVCN shell produced substantial DPOAE effects, whereas stimulation in DCN or AVCN core were ineffective (Figure 6). This comparison was made based on the 80th percentile DPOAE effects.
Fig. 6. Summary of DPOAE effects by CN subdivisions: stimulation sites in PVCN, DCN, AVCN core and AVN shell. Stimulation in PVCN and AVN shell was effective in causing DPOAE effects, particularly in the ear ipsilateral to the stimulated CN, whereas stimulation in DCN and AVN core was ineffective in either ear. The comparison was based on the 80th percentile value of DPOAE effects, obtained with interpolation from each CN subdivision.

from data set of each CN subdivision (Figures 3 and 4A).

In both PVCN and AVN shell, DPOAE effects measured in the ear ipsilateral to the stimulated CN were larger. Using the 80th percentile value, ipsilateral effect was 3.7 dB stronger in PVCN, and 11.4 dB in AVN shell (Figure 6).

3.4 Discussion

3.4.1 MOC interneurons in PVCN Electric stimulation in PVCN caused significant changes in DPOAE (Figures 3, 6 and 7). We interpret this as an activation of the MOC reflex, providing evidence that PVCN contains MOC interneurons. This is consistent with anatomical findings that PVCN neurons project to MOC neurons (Thompson and Thompson 1991; also see Warr 1969), and also consistent with a physiological study (de Venecia et al. 2005), wherein PVCN lesions abolished acoustically evoked MOC reflex. The present study demonstrated that MOC interneurons in PVCN have excitatory action on MOC reflex.

Within PVCN, many effective sites were located dorsomedially (Figure 7). Similarly, particularly effective lesions were located in the dorsomedial PVCN (de Venecia et al. 2005). This indicates that MOC interneurons are located in this area, but does not preclude additional MOC interneurons in more ventral PVCN. Interpretation of data from ventral PVCN would be subject to technical difficulties because MOC in-
Summary of stimulation sites and DPOAE effects shown on CN atlas. Top row contains stimulation sites with contralateral measurements, and the bottom row, ipsilateral. The sections are arranged, from the most posterior in the left to the most anterior in right, in ascending A-P scale. A ♠ indicates a site with large DPOAE effects, followed by ●, with progressively less DPOAE effects, and X, representing no or little effects. These symbols were determined by an empirical monotone increasing function of relative shock levels and the DPOAE effect. (E.g., two sites that produce the same DPOAE effect may be classified with different symbols depending on the relative shock levels required to produce it.)
terneurons in dorsal PVCN may send their axons through the ventral PVCN towards VAS, wherein ventral sites may stimulate the axons of passage from dorsally located neurons. Another possibility is that the MOC interneurons may be tonotopically organized like other VCN neurons (Rose et al. 1959; Godfrey et al. 1975; Bourk et al. 1981), where high CF units are located dorsally, and low CF ventrally. We used primary tones of high frequency because they evoke large DPOAE, which may have biased our assay to be most sensitive to high CF MOC interneurons.

3.4.2 MOC interneurons in AVCN shell   Electric stimulation in AVCN shell caused significant changes in DPOAE from the ipsilateral ears (Figures 4, 6 and 7) indicating that AVCN shell neurons can activate MOC reflex. This is consistent with anatomical findings that AVCN shell neurons project to MOC neurons (Ye et al. 2000). The present study demonstrates that the AVCN shell neurons provide an excitatory action on MOC neurons.

The AVCN shell pathway is unlikely to play a primary role in sound evoked MOC reflex, at least at low sound levels, since a small kainic acid lesion in PVCN is sufficient to abolish acoustically evoked MOC reflex (de Venecia et al. 2005). Another reason is the physiological response properties of shell neurons. Single unit study of AVCN shell neurons indicated that neurons that respond to sound can have non-monotonic firing rates with increasing stimulus level, tuning to multiple frequencies, inhibitory frequency areas with PSTH of on, pauser or unusual types, and rather low maximum firing rates with noise stimuli (Ghoshal and Kim 1997). In contrast, single unit recording of MOC axons showed frequency tuning slightly wider than primary auditory nerve, with vigorous response to noise stimuli, with chopper-like peristimulus histogram (PSTH) (Liberman and Brown 1986). Such responses of MOC neurons are unlikely derived from inputs from AVCN shell neurons, thereby making AVCN shell an unlikely candidate for interneurons of sound-evoked MOC reflex. Although the AVCN shell neurons may not function as the reflex interneurons, our study has demonstrated that their inputs to MOC neurons have a powerful influence.

DPOAE effects were larger in the ipsilateral ear in general, but AVCN shell stimulation appeared to have more pronounced ipsilateral preference (Figure 6). Compared at the 80th percentile values, DPOAE in ipsilateral ear was larger with stimulation in AVCN shell than PVCN, but DPOAE in contralateral ear was larger with stimulation in PVCN than AVCN shell. This leads to one possibility that shell interneurons may project preferentially to MOC neurons that project to the ear ipsilateral to the stimulated CN. In one available case, DPOAE was measured in the ear ipsilateral to the stimulated CN, while surgically cutting decussating MOC axons at the floor of 4th ventricle. The DPOAE effect decreased from 6.2 dB to 2.7 dB. The reduction in the DPOAE effect is attributable to the interrupted double-crossed pathway, and the residual effect uninterrupted uncrossed pathway. This is consistent with Ye et al. (2000) where the ipsilateral-to-contralateral ratio of AVCN shell neurons projecting to MOC was 52:29 in cat.

Our findings are consistent with the hypothesis that AVCN shell may be involved
in suppression of self-generated sounds such as respiration, chewing or vocalization. AVCN shell receives inputs from cuneate nucleus (Itoh et al. 1987; Weinberg and Rustioni 1987), and somatosensory neurons in trigeminal ganglion (Shore et al. 2000; Shore and Zhou 2006). Electric stimulation of the trigeminal somatosensory neurons inhibit sound-evoked responses in CN neurons (Shore and Zhou 2006). The axodendritic terminals of trigeminal sensory neurons in AVCN shell contain small round vesicles (Shore et al. 2000), indicating that the pathway is presumably excitatory. Electric stimulation in AVCN shell excites MOC reflex (present study). Taken together, excitation of trigeminal somatosensory neurons may decrease the cochlear sensitivity.

3.4.3 MOC activation by VAS stimulation Stimulation in some sites in VAS produced large DPOAE effects (Figures 4 and 5). This is probably because the MOC interneurons send their axons through VAS. In support of this idea, severing IAS and DAS did not significantly alter the DPOAE effect (Figure 7).

3.4.4 Limitations of this approach for studying interneurons of the MOC reflex Using our technique, DPOAE effects were interpretable if the DPOAE level changes significantly, but not absence of changes. Deeter et al. (2009) and Siegel and Kim (1982) showed that DPOAE level may increase, decrease or not change, when the MOC reflex is activated, depending on the choice of primary tone frequencies and levels. In addition, MOC interneurons may be organized tonotopically within CN, and a stimulation site may activate the MOC interneurons in only a certain frequency range. Therefore, we cannot interpret the absence of a DPOAE change as the absence of activation of the reflex.

Finally, the size and shape of the bipolar electrode used are suboptimal to stimulate surface structures, such as AVCN shell and DCN. This may be one reason for little or no effects at some sites in AVCN shell.

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DPOAE effects from electric stimulation of cochlear nucleus
Bibliography


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