

The effect of smooth muscle antagonists on the sound-induced motion of the tympanic membrane

By

Amanda J. Graves

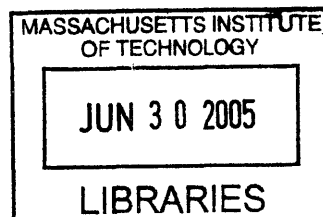
B.A. Arts and Sciences
The Ohio State University, 2002

SUBMITTED TO THE DEPARTMENT OF HEALTH SCIENCES AND TECHNOLOGY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE IN HEALTH SCIENCES AND TECHNOLOGY
AT THE
MASSACHUSETTS INSTITUTE OF TECHNOLOGY

JUNE 2005

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Signature of Author: _____
Amanda J. Graves
Harvard-MIT Division of Health Sciences and Technology
May 16, 2005

Certified by: _____
John Rosowski
Member of the Faculty of Health Sciences and Technology, HMS, MIT
Thesis Supervisor

Accepted by: _____
Martha L. Gray, Ph.D. / Edward Hood Taplin
Professor of Medical and Electrical Engineering /
Director, Harvard-MIT Division of Health Sciences and Technology

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Abstract

The *pars tensa* of the tympanic membrane is composed of three layers: an epidermal layer, a fibrous layer, and a mucosal layer. Recent studies (Kuijpers et al, 1999; Henson and Henson, 2000; Henson et al, 2005) suggest that the fibrous layer in several mammalian species contains contractile fibers, which are located primarily within the thickened border of the *pars tensa* known as the annulus fibrosis. These contractile fibers resemble smooth muscle fibers.

Yang and Henson (2002) studied the physiological effects of pharmacological modulators on the *pars tensa* of the annulus fibrosis by measuring the sound-induced cochlear response. Their results suggest a dose-dependent change in cochlear response after application of sodium orthovanadate and norepinephrine. Application of saline induced no change in cochlear response. Based on their data, Yang and Henson proposed that the pharmacological agents altered the function of the smooth muscle fibers of the annulus fibrosis to produce a mechanical change in the tympanic membrane.

In this study two measurements, cochlear response and Laser Doppler Vibrometry, were used to assess the sound-induced velocity of the tympanic membrane of the gerbil before and after application of saline and varying concentrations of three smooth muscle antagonists (sodium orthovanadate, norepinephrine, and carbachol) to the *pars tensa*. It was demonstrated that applications of saline and varying concentrations of sodium orthovanadate were associated with both increases and decreases in the magnitude of the cochlear response in two out of three ears tested. There was no evidence of a dose-dependent change in the cochlear response. Applications of saline and varying concentrations of sodium orthovanadate, norepinephrine, and carbachol were associated with increases and decreases in the magnitude of the Laser Doppler Vibrometry response in eight of fourteen ears tested. Evidence of a dose-dependent change in Laser Doppler Vibrometry results was obtained in one ear. The results of this study suggest that application of any substance to the tympanic membrane may or may not be associated with an increase or decrease in the cochlear response or Laser Doppler Vibrometry response, and thus, the source of mechanical changes observed at the tympanic membrane is not necessarily the smooth muscle fibers of the annulus fibrosis.

Acknowledgements

Due to a set of challenging, yet extraordinary, circumstances, the beginning of my Ph.D. work has turned into this Masters thesis. Although I would like to thank everyone who has stood by me over the last three years, there are a few people I must single out for their unlimited patience, support, and time.

First, I would like to thank my mentor, John Rosowski. John, thank you so much for the opportunity to work with you over the last year. I don't know how you did it, but when most people would have given up on me, you found something in me to believe in. Your unlimited patience, support, expertise, and time have been invaluable to me and to my career at MIT--for this I am forever grateful.

I would also like to thank fellow lab members Jocelyn Songer, Mike Ravicz, Howard Chan and Melissa Wood. Jocelyn, Mike and Howard, thank you for all of your advice while I was reading literature, conducting experiments, and writing this thesis. Melissa, thank you for your help and surgical expertise.

Lastly, thank you to my Mom, my Dad, and my brother, Ron. I have had to make a lot of life-changing decisions over the last three years and you have done nothing but give me encouragement and support EVERY step of the way. You are my family, my best friends, and my support system, and therefore, I dedicate this to you. I love you.

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1. Introduction

We are surrounded by sounds in our everyday environment. Whether we are exposed to environmental noises, conversation, music, or other sounds, the sense of hearing makes a profound impact on the lives of normal hearing individuals. In order to understand how we hear, it is important to understand how the different structures of the ear work individually and together to transmit the acoustic signal from the surrounding environment to the central auditory system. The processes within the auditory periphery are central to this transmission process.

The structures in the auditory periphery are very sensitive. The auditory system can detect motions of the eardrum, a structure in the middle ear, that are on the order of the diameter of a hydrogen atom. Part of this sensitivity comes from amplification and sound transformation performed by the middle ear. Mechanical movements and the size of structures within the middle ear allow for effective transmission of sound from the air in the outer ear to the cochlear fluid in the inner ear. Mechanical movements of 1) the eardrum, 2) a chain of three bones, and 3) two small skeletal muscles effect transmission of sound through the middle ear. Recently, contraction of smooth muscle fibers located in the periphery of the eardrum has been hypothesized to affect the path of sound transmission through the middle ear. The research presented in this thesis examines the mechanical function of these smooth muscle fibers.

In this section, a general introduction to the anatomy of the peripheral auditory system will be provided, followed by a brief description of what we know about the process of sound transduction. The anatomy of the tympanic membrane (commonly known as the eardrum) will then be discussed and the possible functions of the anatomically distinct sections of the tympanic membrane will be explored. The specific

research question this thesis examines, and the research that gave rise to it, will then be introduced.

1.1 Anatomy of the Peripheral Auditory System

The ear (Figure 1.1) is grossly divided into three sections: the outer ear, the middle ear, and the inner ear. The outer ear consists of three easily identifiable structures: the pinna, the concha, and the external auditory canal. The pinna is the large visible flap of the outer ear. The deeper, bowl-like portion of the ear that is surrounded by the pinna is referred to as the concha. At the bottom of the concha is an opening that leads to the nearly cylindrical external auditory canal. The external auditory canal is terminated medially by the tympanic membrane (TM). The TM is the end of the outer ear and the beginning of the middle ear (Shaw, 1974).

The middle ear is an air-filled space that contains a chain of three tiny bones, the malleus, incus, and stapes. These bones are collectively referred to as the ossicular chain. The manubrium (or handle) of the malleus is attached to the medial side of the tympanic membrane by a network of fibrous connective tissues within the TM. The head of the malleus is connected to the body of the incus by the incudo-malleolar joint, and the incudo-stapedial joint connects the lenticular process of the incus to the head of the stapes. The stapes footplate rests in a small hole in the bony case of the inner ear called the oval window. The stapes footplate marks the end of the middle ear and the oval window marks the beginning of the inner ear (Henson, 1974).

There are four other important structures located in the middle ear space. First, as mentioned previously, the middle ear is an air-filled space. It is the compressibility of this air cushion that allows an impinging sound to set the TM into motion. Second, the

Eustachian tube, a tube that connects the middle ear space to the nasopharynx, periodically opens by swallowing or yawning in order to maintain the air in the middle-ear space at ambient pressure. Lastly, the middle ear contains 2 muscles: the tensor tympani muscle and the stapedius muscle. The tensor tympani muscle emerges from the Eustachian tube and attaches superiorly to the manubrium of the malleus via a tendon. The stapedius muscle is located within the bony canal that encases the facial nerve. A short tendon attaches the stapedius muscle to the head of the stapes (Rosowski, 1994).

The third part of the ear, the inner ear, is a fluid-filled space separated from the middle ear by the oval window. The auditory sensor within the inner ear is the cochlea, a snail-shaped, fluid-filled structure embedded in the temporal bone. The cochlea can be divided into fluid compartments: the scala vestibuli, the scala tympani, and the scala media. The scala vestibuli and scala tympani are connected at the apex of the cochlea by the helicotrema. The oval window and the vestibular apparatus bound the scala vestibuli laterally. The scala tympani is bounded laterally by the round window, another small hole in the petrous bone that is covered by a membrane. The round window is positioned inferiorly, and somewhat anteriorly, to the oval window in the cochlear wall. The third fluid compartment, the scala media, separates the other two compartments over much of their length. The scala media is completely isolated from the other two compartments: the boundary between the scala vestibuli and the scala media is Reissner's membrane, and the boundary between the scala tympani and the scala media is the basilar membrane. The scala media contains the Organ of Corti, which is the organ of hearing. The Organ of Corti contains the auditory sensory cells (Corti, 1851). These sensory cells perceive sound-induced motions in the cochlear partition created by the coupling between the

stapes and the oval window (Helmholtz, 1885). The sensory cells chemically communicate with auditory nerve fibers, which are located just inferior to the sensory cells.

1.2 Sound Transmission

Sound can be transmitted through any elastic medium such as air or water. As a sound source vibrates, the air molecules around the source are set into motion and these motions set adjacent layers of molecules into motion. The air molecules themselves do not move from the source to the receiving object, but rather, it is the physical forces and motions propagating through the medium that are passed from layer to layer of air molecules until the vibration reaches an object such as the ear. This vibration pattern is often characterized as a series of pressure changes in the transmitting medium.

As a sound impinges on a listener, the sound is scattered and diffracted by the head, shoulders, and the pinna. These structures change the characteristics of the sound before it enters the ear in a manner that is dependent on the direction of the sound source. Some fraction of the scattered and diffracted sound enters the ear and travels down the external auditory canal (Shaw, 1974).

The sound in the external auditory canal can be transmitted to the inner ear via three different paths (Merchant and Rosowski, 2003): 1) bone conduction where the sound skips the middle ear and is transmitted directly to the inner ear through bone, 2) variations in sound pressure in the air of the middle ear air space, and 3) the ossicular chain in the middle ear space. The third path is the most effective path for sound transmission in the normal ear and is detailed below.

The TM vibrates in response to the impinging pressure variations from the external auditory canal, thus the sound is transformed from an acoustic stimulus to a mechanical stimulus. As the TM vibrates, the vibration is transmitted to the ossicular chain through the attachment between the malleus and TM. As the ossicular chain vibrates, the stapes footplate in the oval window vibrates, which causes a fluid disturbance in the scala vestibuli of the cochlea. Because the cochlea is embedded in the effectively rigid temporal bone that is open only at the two windows, and because the cochlea is filled with “nearly” incompressible fluid, the inward motions of the stapes at the oval window are accompanied by outward motions of the round window. The associated “nearly” instantaneous pressure wave in the cochlear fluid produces a sound pressure gradient along the cochlear partition that gives rise to a traveling wave along the length of the basilar membrane. The motion of the basilar membrane stimulates the auditory sensory cells. The stimulated sensory cells release chemical neurotransmitters that induce action potentials in the auditory nerve. These neural signals are transmitted along auditory nerve fibers to structures in the central auditory pathway (Dallos, 1996).

1.3 Anatomy of the TM

Sound-induced motions of the TM are the first step in the conversion of sound in the ear canal to mechanical vibrations in the middle ear. The size, thickness, and structure of the TM vary greatly among species; however, the TM in all species can be grossly divided into two sections: the *pars flaccida* and the *pars tensa* (Figure 1.2).

Shrapnell (1832) first described the *pars flaccida* in mammals as being anatomically distinct from the *pars tensa*. In humans, the *pars flaccida* is a small triangular and flaccid portion of the TM that lies above the malleolar folds and is attached

directly to the petrous bone. Sound-induced motions of the *pars flaccida* are independent of ossicular motion (Teoh et al., 1997; Rosowski et al., 1997). Shrapnell (1832) described the *pars flaccida* as having three distinct layers: a lateral epidermal layer, a medial mucosal layer, and a middle fibrous layer. Lim (1968b) found that the middle fibrous layer of the *pars flaccida* in the cat, squirrel monkey, rabbit, and sheep contains a higher proportion of elastic fibers compared to inelastic (i.e. collagen) fibers. These fibers are not anatomically ordered within the fibrous layer of the *pars flaccida*.

The *pars tensa*, what most people consider to be the eardrum, is coupled to the temporal bone via an annulus of connective tissue. In most species, the *pars tensa* is a curved structure and is generally larger in area than the *pars flaccida*, though there are some exceptions to this rule (Kohloffel, 1984). Lim (1968a) studied the microscopic morphological details of the *pars tensa* of the guinea pig, cat, and squirrel monkey. In these species the *pars tensa* has three layers: a lateral epidermal layer, a medial mucosal layer, and a stratified middle layer called the lamina propria (Lim, 1968a). The lamina propria of the *pars tensa* is structurally more complex than the middle fibrous layer of the *pars flaccida*. The *pars tensa* is tightly coupled to the manubrium of the malleus via the fibers of the lamina propria. Lim (1968a) divided the lamina propria into four ordered layers: 1) the subepidermal connective tissue layer, 2) the radiate collagenous bundle layer, 3) the circular collagenous bundle layer, and 4) the submucosal connective tissue layer. While the details of these layers are not important for the purpose at hand, it should be noted that an extensive network of associated unmyelinated nerve fibers, capillaries, and fibroblasts were found in some of the lamina propria layers. A cross-sectional view of the *pars tensa* of a squirrel monkey is shown in Figure 1.3.

Recently, Kuijpers et al. (1999) and Henson and Henson (2000a) have examined the microstructure of the annulus fibrosis, which is the thickened circumferential border of the *pars tensa* that connects the membrane to the tympanic bone. Kuijpers et al (1999) found that the annulus fibrosis of the rat contained myofibroblasts, which have contractile properties. Henson and Henson (2000a) found the annulus fibrosis of the mustached bat to have a highly specialized myovascular zone composed of smooth muscle fibers, which seemed to be associated with the fibers of the lamina propria (Figure 1.4). Both types of contractile elements (i.e. myofibroblasts and smooth muscle fibers) are in the same location in both species.

Based on micro-anatomical studies, Henson et al. (2005) (also see Henson and Henson, 2000a,b; Henson et al., 2001a,b, 2002) concluded that smooth muscle in the annulus fibrosis was a common feature among the following mammalian orders: insectivora (e.g. the European hedge hog), Chiroptera (e.g. the pallid bat), Rodentia (e.g. the Mongolian gerbil, Laboratory rat, and Laboratory mouse), and Primates (e.g. human). Although differences between the mammalian orders were found, the smooth muscle fibers in all species examined appeared to form a rim of contractile elements in the annulus fibrosis of the *pars tensa*. Furthermore, the smooth muscle fibers were found to be associated with both unmyelinated nerve fibers and vascular structures within the annulus fibrosis in all species.

1.4 Function of the Pars Flaccida

The function of the *pars flaccida* is currently under investigation by several researchers. The high proportion of elastic fibers of the *pars flaccida* appears to allow it to move independently from the *pars tensa* (Rosowski et al., 1997; Teoh et al., 1997).

Original theories about the function of the *pars flaccida* suggest that this structure was involved in equalizing static pressure in the middle ear space by acting as a volume buffer during gas absorption (Shrapnell, 1832; Stenfors et al., 1979). More recent studies, however, suggest the *pars flaccida*'s role in pressure equalization in the sound transduction process is rather small (Hellstrom and Stenfors, 1983; Dirckx et al., 1998; Rosowski and Lee, 2002). Others have suggested that the role of the *pars flaccida* is to limit the mechanical response of the middle ear to low-frequency sound stimulation (Kohloffel, 1984; Rosowski et al., 1997). Studies conducted using an acoustic model of the ear (Kohloffel, 1984) suggested that the *pars flaccida* acts as a shunt that reduces the sound-induced motion of the *pars tensa* at low frequencies. Physiological measurements of sound-induced *pars flaccida* and *pars tensa* motions in gerbils are consistent with such a low-frequency shunt (Teoh et al., 1997; Rosowski et al., 1997).

1.5 Function of the Pars Tensa

The *pars tensa* plays an important role in sound transmission from the outer ear to the middle ear and from the middle ear to the inner ear. As a sound impinges on the TM, causing the TM to vibrate, the sound energy is changed from acoustic energy to mechanical energy. A portion of this mechanical energy is directly transferred to the ossicular chain via the tight coupling between the *pars tensa* and the manubrium of the malleus (Tonndorf and Khanna, 1970). Decraemer et al. (1991) further examined the relationship between this coupling. They found that the frequency-dependent malleus vibration modes are related to the coupling between the *pars tensa* and the manubrium, and thus, to the vibration pattern of the tympanic membrane. Furthermore, Helmholtz (1868, translated 1873) and Tonndorf and Khanna (1970) found that the curved shape of

the *pars tensa* was a crucial part of the middle's ear acoustic-to-mechanical transformer mechanism. Dirckx and Decraemer (2001) found that the shape of the *pars tensa*, as well as the TM as a whole, at positive pressures is largely dependent on the presence of the malleus. Thus, the *pars tensa*, and its tight coupling to the manubrium of the malleus, is directly involved in sound transduction from the outer ear through the middle ear to the inner ear.

1.6 Feedback control of the middle ear: The middle ear muscles

Research suggests that the stapedius muscle and the tensor tympani are functionally different. Politzer (1861) first observed that motor innervation for these two muscles is from two different cranial nerves: the stapedius muscle is innervated by the facial nerve and the tensor tympani is innervated by the trigeminal nerve. Early studies (Wiggers, 1937; Moller, 1964, and Borg, 1968 and 1972) suggest that the main function of the middle-ear muscles is to protect the inner ear from intense acoustic stimulation by attenuating the sound before it reaches the oval window. More recent studies (Borg et al., 1984; Pang and Guinan, 1997), however, suggests that this attenuation helps to improve perception by decreasing masking of high-frequency stimuli in the presence of low-frequency noise. Whether the middle-ear muscles serve to protect the inner ear, reduce the effects of masking, or both, the muscles must receive feedback control from a higher level.

Borg (1973) used degeneration techniques to locate the motor neurons of the stapedius muscle. The study found that the stapedius muscle neurons are located in the facial nucleus. Other studies (Strutz et al., 1988; Joseph et al., 1985), however, concluded that stapedius muscle neurons are located just outside of the facial nucleus.

Furthermore, Joseph et al. (1985) found that some neurons may extend to within the superior olivary complex. These motor neurons respond to contralateral, ipsilateral, and bilateral stimulation as well to tonic activity (Kobler et al., 1987; Vacher et al., 1989). The response properties of these neurons support the hypotheses that the stapedius muscle may be instrumental in protecting the inner ear, reducing the effect of low-frequency masking, or both.

Studies (Mizumo et al., 1982; Strutz et al., 1988) discovered tensor tympani motor neurons are located just outside the trigeminal motor nucleus. In the cat, Simmons (1962) found that the tensor tympani muscle contracts during acoustic stimulation at very high levels. A different suggestion came from Klockhoff (1961) who showed that acoustic stimulation failed to elicit a contraction of the tensor tympani. Borg and Zakrisson (1975), however, concluded that the tensor tympani in humans only contracts in response to self-vocalization. Because it is not understood clearly what stimuli, if any, elicit the contraction of the tensor tympani, the actual function of the tensor tympani is still widely speculated. It is, however, the only muscle capable of moving the TM due to the tensor tympani's connection to the manubrium of the malleus that is tightly coupled to the *pars tensa* of the TM.

1.7 Role of tension in the Pars Tensa

The recent anatomical discovery of smooth muscle fibers in the annulus fibrosis of several mammalian species has introduced another possible control of sound transformation via the *pars tensa*. Kuijpers et al. (1999), Henson and Henson (2000) and Henson et al. (2005) suggest that the unique peripheral positioning of these smooth muscle fibers or myofibroblasts, and their association with fibers in the lamina propria of

the *pars tensa*, coupled with the presence of unmyelinated nerve fibers and vascular networks near the smooth muscle fibers, make these fibers prime candidates to regulate blood flow and/or to create and maintain tension of the tympanic membrane.

To test this hypothesis, Yang and Henson (2002) applied smooth muscle antagonists (i.e. substances that cause smooth muscle fibers to contract) and smooth muscle relaxers to the *pars tensa* of the tympanic membrane in gerbils. The drugs' effects on the sound level threshold necessary to produce a cochlear microphonic (CM) of 4 μ volt were measured. The CM is an extracellular measure of cochlear function. The premise behind measuring CM is: If application of smooth muscle antagonists caused the smooth muscle fibers in the *pars tensa* to contract, such a contraction should increase the tension in the TM. Such an increase in tension of the TM would increase the stiffness of the TM and the ossicular chain, which would reduce sound transmission to the inner ear much like the effect of contraction of the stapedius muscle.

When smooth muscle antagonists were applied to the TM, Yang and Henson (2002) observed concentration-dependent increases in CM threshold (decrease in response). Application of 10mM sodium orthovanadate (Figure 1.5), a smooth muscle antagonist, caused a maximal increase of 7-9 dB in CM threshold at 2.16kHz, the lowest frequency tested. The increases in CM threshold at 4.47 kHz and 9.11 kHz were less than the increase at 2.16 kHz, although still significant. Concentrations above 10mM induced no further increase in CM threshold. Norepinephrine, a catecholamine hormone known for its vascular effects on smooth muscle (Ahlquist, 1948), produced smaller, although similar, frequency-dependent results compared to sodium orthovanadate. The magnitude of CM threshold increase for norepinephrine was 3 dB at 2.16kHz, which is

less than the 7-9 dB increase in threshold induced by sodium orthovanadate at 2.16kHz. It has been proposed that sodium orthovanadate not only induces smooth muscle contraction, but may also increase the force of smooth muscle contraction via stimulation of the tyrosine kinase pathway (Alcon et al., 2000). This may explain the larger effect of sodium orthovanadate. The demonstration of norepinephrine effects suggests the presence of adrenergic receptors on the smooth muscle cells. The increase in CM threshold associated with both sodium orthovanadate (Figure 1.5) and norepinephrine application was reversible with saline washes.

Yang and Henson also examined the effect of applying smooth muscle relaxers to the TM. After a previous saline wash, the introduction of smooth muscle relaxers (e.g. sodium nitroprusside and calcium channel blocker) to the system produced either no change in CM threshold, a slight decrease in CM threshold, or an oscillating CM threshold (± 1.0 dB) with regard to baseline measurements (Yang and Henson, 2002). However, if smooth muscle relaxers were applied after application of a smooth muscle antagonist, the CM threshold returned to pre-smooth muscle antagonist application values. The reversal of CM threshold using smooth muscle relaxers was approximately 10 minutes, which is much faster than the 30-minute reversal produced by saline washes (Figure 1.6).

Lastly, Yang and Henson (2002) applied sodium orthovanadate to the round window of the cochlea and to the tensor tympani muscle of the middle ear. The results of these two control experiments were compared to the results of sodium orthovanadate placement on the external surface of the *pars tensa*. Application of a high concentration (20.0mM) of sodium orthovanadate to the round window caused a 7-10 dB increase in

CM threshold, similar to the change in CM threshold observed when sodium orthovanadate was applied to the *pars tensa* (Figure 1.7). The time required for changes in CM threshold to be observed was much faster when sodium orthovanadate was placed on the TM (i.e. 5 minutes) versus when it was placed on the round window (i.e. 40-60 minutes). Furthermore, the CM threshold shift was greatest at the lowest frequency tested (2.16 kHz) when sodium orthovanadate was applied to the *pars tensa*, but the CM threshold shift was greatest at the highest frequency tested (9.11 kHz) when sodium orthovanadate was applied to the round window. When sodium orthovanadate was applied to the tensor tympani muscle fibers, there was no significant change in CM threshold. The results of both experiments suggest that the substances applied to the TM were only acting upon the smooth muscle fibers of the *pars tensa* and not on other structures in the peripheral auditory mechanism.

From the results above, Yang and Henson (2002) considered that the function of the smooth muscle fibers in the annulus fibrosis is to maintain tension of the *pars tensa* and/or to regulate and control blood flow within the TM where changes in engorgement affect the TM mechanism. If the smooth muscle fibers do maintain tension of the *pars tensa*, this may be a third mechanism (along with the two middle ear muscles) for the feedback control of energy levels reaching the inner ear. A smooth muscle controlled mechanism might be expected to be slower in onset compared to contraction of the tensor tympani or stapedius muscle; however, a smooth muscle controlled mechanism is more capable of sustained contraction compared to the striated muscles.

1.8 Goals of this Thesis

The primary goal of this thesis is to investigate the hypothesis that a smooth muscle system controls the tension of the *pars tensa* (Yang and Henson, 2002). The means we will use is to look at the effect of smooth muscle antagonists on an acoustic mechanical correlate of sound transduction, the sound-induced motion of the malleus. We reason that the changes in TM tension that affect sound transmission should have a primary effect on the mechanical motion of the *pars tensa* and the ossicles. A secondary goal of this thesis is to attempt to confirm the results obtained by Yang and Henson (2002).

2. Materials and Methods

2.1 Cochlear Microphonic (CM) versus Cochlear Potential (CP)

A basic technique to determine cochlear stimulation is to place a wire electrode near the round window of the cochlea and measure the sound-evoked electrical potential. Yang and Henson (2002) used this technique to measure what they called the cochlear microphonic (CM). A similar technique was used in the work reported here. However, in our laboratory it is understood that these auditory potentials are complex in origin including AC sensory potentials (the true cochlear microphonic), DC sensory potentials (sometimes called summing potentials), and averaged neural activity (the compound action potential). Because of these complex origins, I will refer to sound-evoked potentials I measure as the cochlear potential (CP).

2.2 Animals Used

We chose to study the TM of the Mongolian gerbil (*Meriones unguiculatus*) because 1) the TM is relatively large, 2) the surgical exposure is relatively easy, and 3) the results in Yang and Henson (2002) were obtained in the gerbil ear. Measurements were made in healthy female Mongolian gerbils of normal weight (60-80 grams). Fourteen ears of twelve gerbils were used for data analysis. The Animal Care and Use Committee at the Massachusetts Eye and Ear Infirmary (MEEI) and the Massachusetts Institute of Technology (MIT) approved the protocol for this study.

2.3 Animal Preparation

All surgeries were performed in the surgery room in the Eaton-Peabody Laboratory (EPL) at MEEI. Two anesthesia regimens were used. In earlier experiments, initial doses of sodium pentobarbital (Nembutal) of 40mg/kg body weight and Ketamine of 25mg/kg body weight were administered ten minutes apart followed by alternating half-doses of the two drugs until the animal was fully sedated (i.e. no hind leg withdrawal was observed upon pinching the foot). Half-doses of both drugs were given throughout the duration of the experiment to maintain sedation of the animal. During some experiments, however, severe respiratory and cardiac problems were associated with the anesthesia protocol. In later experiments, baseline doses were changed to alternating doses of Nembutal (40mg/kg body weight) and Ketamine of 50mg/kg body weight.

After the gerbil was anesthetized, the undersurface of the gerbil's neck and the head around both ears were shaved. The gerbil was placed on a heating pad and the body temperature was monitored throughout the entire experiment. Body temperature was maintained between 34°C and 40°C.

A tracheotomy with cannulation was performed. The pinna and cartilaginous portion of one ear were removed, which left only the bony ear canal intact. Muscle tissue and tendons located superiorly, posteriorly, and inferiorly to the gerbil's bony ear canal were removed to expose the posterior bulla and the inferior lateral portion of the bony ear canal. A small hole was placed in the superior posterior chamber of the bulla and a vent was placed in the hole to aerate the middle ear during the experiment. The vent was sealed into place with dental cement. Another hole was placed in the inferior-posterior

chamber of the bulla. This hole gave access to the round window and was covered with a saline-soaked cotton ball until the cochlear potential (CP) electrode was positioned.

The inferior lateral portion of the bony ear canal was partially removed to provide a better view of the *pars tensa*. The black, melanocyte-rich skin layer of the bony ear canal was entirely removed. Removal of this skin layer allowed future application of drugs to reach the smooth muscle fibers of the annulus fibrosis.

Lastly, a small piece of reflective material consisting of three-to-five 50-micron reflective beads on a piece of tape was placed on the umbo of the manubrium near the center of the *pars tensa*. The reflective material was needed to generate enough reflected laser light from the umbo to ensure accurate measurement of umbo velocity by the Polytec laser vibrometer. The reflective material was of small enough size and mass that it had no effect on the sound-induced measurements of the TM motion. The animal was moved to Chamber VI in EPL to continue with experimental set-up and measurements.

In Chamber VI, the gerbil was coupled to an EKG to monitor heart rate throughout the procedure and then a small brass coupler containing a side-mounted microphone and sound tube was placed at the entrance to the bony ear canal. The placement of the brass coupler had to provide (a) a reasonable view of the TM for drug application and (b) a clear path for the laser to be focused on the reflective material located on the umbo. Once these two criteria were met, the brass coupler was sealed to the bony ear canal using Geltrate®. Lastly, a small wire electrode was positioned on the bone surrounding the round window in the middle ear cavity through the inferior posterior hole in the bulla to measure cochlear potential (CP). Once a CP was obtained in

response to vocal stimulation, the electrode was secured in place with dental cement. The experimental set-up is shown in Figure 2.1.

2.4 Instrumentation

The high frequency measurement system written in Labview (version 7.1) software was used to generate a train of broadband log chirps (11Hz-24kHz) with a low-frequency pre-emphasis. The duration of the log chirp was 0.09 seconds. The log chirp was downloaded to a digital-to-analog Hewlett Packard (33120A) waveform generator. The generator output the signal to an anti-aliasing filter with a cut-off frequency of 24 kHz. The signal was passed through a TDT (i.e. Tucker Davis Technologies) PA5 USB bus-controlled Attenuator and a Crown D-75 Power Amplifier to a Beyer DT-48 earphone.

A Knowles (3027) microphone calibrated against a standard reference microphone was used to measure the stimulus sound pressure in the ear canal. A 9-V battery supplied the microphone voltage. A Grass amplifier amplified the microphone signal before being measured by one channel of the Labview controlled D/A converter. A second Grass amplifier amplified the CP response. Either the CP or the output signal from the Polytec laser vibrometer were measured by a second channel of the Labview controlled D/A converter. The microphone signal was amplified by 40 and the CP response was amplified by 333 during most measurements.

2.5 Smooth Muscle Antagonists

In this study we used three compounds that are known to cause smooth muscle contraction: sodium orthovanadate (NaOV), norepinephrine (NEP), and carbamylcholine

chloride, otherwise known as carbachol (CRB). Small quantities (10 μ l) of all three compounds were applied to the *pars tensa* by one of two methods: 1) pipette, or 2) micro-syringe. The likelihood of cross-contamination between different drugs or different concentrations of the same drug was very low. The pipette tip was replaced with a new tip after every drug and saline application. The syringe was washed four-to-five times with saline before applying another concentration of the same drug or before applying a different drug.

The use of a pipette and syringe to deliver the saline and the smooth muscle antagonists to the *pars tensa* was problematic. The tip of the delivery instrument needed to be close to the TM to ensure proper drug application. It was easy, however, to get too close to the TM such that the instrument would puncture the TM. Approximately 50% of all experiments were terminated by TM puncture.

2.5.1 Sodium Orthovanadate

NaOV and other vanadium compounds are known to relax smooth muscle at low concentrations and to contract smooth muscle at high concentrations. In the isolated human bronchus, Cortijo et al. (1997) found that vanadium compounds act directly on smooth muscle by elevating cytosolic free calcium through promoting release of intracellular calcium. Vanadium compounds are also inhibitors of phosphotyrosine phosphate activity (Swarup et al., 1982). A recent study (Alcon et al., 2000) has concluded that the effect of vanadate on smooth muscle in the gallbladder of the guinea pig is mediated by protein tyrosine phosphorylation. This mediation process could potentially increase the contractile force of smooth muscle contraction. The NaOV used

in this study was obtained from Sigma-Aldrich (product number S6508) in powder form.

Approximately one-half of all NaOV experiments were conducted with an inactivated NaOV solution. The inactivated solution was made by diluting the NaOV powder with HEPES solution to obtain and use at the following concentrations: 0.1mM, 0.3mM, 1.0mM, 3.0mM 10.0mM, and 30.0mM. The other one-half of all NaOV experiments were conducted with an activated NaOV solution (Gordon, 1991) to ensure maximal inhibition of protein tyrosine phosphatases. A 200mM stock solution was prepared. The stock solution was adjusted to pH 10.0 by using 1N sodium hydroxide (NaOH) if the solution was too acidic or 1N hydrochloric acid (HCl) if the solution was too basic. At a pH of 10.0 the solution was yellow. The solution was boiled until it turned colorless (approximately 10 minutes) and then cooled to room temperature. This process was repeated (i.e. readjusted to 10.0, boiled, and then cooled) until the solution stabilized at a pH of 10.0. The solution was then diluted with HEPES buffer to obtain and use at the following concentrations: 0.1mM, 0.3mM, 1.0mM, 3.0mM 10.0mM, and 30.0mM. All NaOV solutions were stored in aliquots at -20°C.

2.5.2 Norepinephrine

NEP is a catecholamine hormone that mediates chemical communication in the sympathetic branch of the autonomic nervous system. The hormone is released from a pre-synaptic nerve fiber to transmit a signal to other nerve cells. It is well known that binding of norepinephrine to smooth muscle cells induces muscle contraction (Ahlquist, 1948). If smooth muscle responds to norepinephrine, this is evidence that adrenergic

receptors are located on the smooth muscle cells. NEP was obtained from Sigma (product number B2296). NEP was diluted in a HEPES buffer to obtain a 10.0mM solution.

2.5.3 Carbamylcholine Chloride AKA Carbachol

Mediation of the parasympathetic branch of the autonomic nervous system is generally mediated by the neurotransmitter Acetylcholine (ACh). Like NEP, ACh is released from a pre-synaptic nerve cell to transmit a chemical message to other nerve cells. ACh promotes free cytosolic calcium in the system, and thus, triggers smooth muscle contraction (Dale et al., 1936). Carbamylcholine chloride (CRB), a choline ester, mimics the action of ACh. CRB was obtained from Sigma (product number C-4382) and diluted in HEPES buffer to obtain a 1.0mM solution. If smooth muscle fibers respond to application of CRB, this is evidence that cholinergic receptors are located on the smooth muscle cells

2.6 Experimental Protocol

Each experiment began with a set of baseline CP measurements and/or laser measurements. Each set of measurements consisted of two or three signal attenuation levels ranging from 20 dBA to 50 dBA. After the baseline series, 10µl of saline was applied to the *pars tensa* of the TM by a manual pipette or syringe. The saline was left on the *pars tensa* for approximately 10 minutes and was then suctioned out. Measurements were made every 5 minutes over the next 20+ minute time period at the different attenuation levels. In three experiments, repeated applications of saline were administered and measurements post-saline application were obtained as described above to test the stability of the system during saline application.

In most experiments, however, after one saline application, 10 μ l of 0.1mM NaOV was administered via a pipette or syringe. Like saline, NaOV was left on the *pars tensa* for approximately 10 minutes and then NaOV was suctioned from the ear. A series of measurements were completed in 5-minute increments over a 20+ minute time period. This whole process was repeated with increasing concentrations of NaOV until the experiment was terminated. In one control experiment, the middle ear space was widely opened to obtain access to the medial mucosal layer of the *pars tensa*. Varying amounts of 10.0mM NaOV were applied repeatedly to the mucosal layer of the *pars tensa* and a series of measurements were made.

During two experiments, 10.0mM of NEP (a high concentration) was applied to the *pars tensa*. NEP was suctioned out and a series of measurements were made over a 20+ minute time period. The whole process was repeated with subsequent doses of 10mM NEP. In one experiment, 1.0mM carbachol was administered to the system. A similar protocol as described for NEP was used.

2.7 Data Analysis

Each stored pair of microphone and laser voltages or microphone and CP voltages were converted to a transfer function in MatLab. The transfer function is the ratio of the measured velocity of the umbo (V_{UMBO}) or CP normalized by the measured sound pressure in the ear canal (P_{EC}). The transfer function is a complex ratio, and therefore, has both magnitude and phase. Only the magnitude of the transfer function, $|CP/P_{EC}|$ or $|V_{UMBO}/P_{EC}|$, was analyzed for this thesis.

The data, once converted to the transfer function, was analyzed via two different approaches. The first approach examined the effects of the saline and smooth muscle

antagonists on the frequency-dependence of the transfer function. The second approach analyzed the time-dependence across the transfer functions.

The CP frequency-dependent baseline response for Gerbil 040726 is shown in Figure 2.2 at three different attenuation levels: 35 dB, 40 dB, and 45 dB. The CP response is non-linear due to non-linearities within the cochlea. In order to minimize the amount of non-linearity within the CP response, an attenuation level was chosen that was 1) low enough to reduce the chance of clipping, or saturating, the CP response, and 2) high enough to minimize noise in the CP response. In Figure 2.2, the attenuation level that best meets these two criteria is 40 dB. At 35 dB of attenuation, there are spikes in the response at low frequencies that are due to clipping of the CP response, and at 45 dB of attenuation, there is a significant amount of noise in the CP response, especially at high frequencies. Thus, the CP response was analyzed at an attenuation level of 40 dB.

The frequency-dependent baseline laser response of Gerbil 040726 is shown in Figure 2.3 at three different attenuation levels: 35 dB, 40 dB, and 45 dB. The response of the middle ear transfer function is linear, and thus, all three attenuation levels were used during data analysis.

The time-dependent magnitude at selected frequencies (i.e. 250 Hz, 500 Hz, 1000 Hz, 2000 Hz, and 4000 Hz) was extracted from the collection of transfer functions. Time was expressed in trial number. In CP experiments, there was approximately 5 minutes between each trial number. In laser experiments, however, the time increment was 5 minutes for every fourth trial number (i.e. there was 5 minutes between Trial 1 and Trial 4, between Trail 4 and Trial 7, etc.).

In order for a response to be considered present, any change in the CP or laser transfer functions must be associated with application of either saline or a smooth muscle antagonist. A transient substance-induced response must be equal to or greater than 3 dB to be considered a response and a sustained substance-induced response must be equal to or greater than 2 dB to be considered a response. The change in response was compared with the set of measurements just prior to the substance application.

3. Results

Results were obtained from twelve gerbils in fourteen ears. CP measurements were obtained from three ears and laser measurements were obtained from all fourteen ears. In only three of these ears were we able to perform a complete dose-response series. Experiments were terminated due to 1) perforations of the TM (most commonly of the *pars tensa* in the vicinity of the umbo), 2) equipment failure, 3) premature death of the animal, or 4) completion of data collection.

3.1 Cochlear Potential Results

A series of baseline measurements were obtained at varying attenuation levels at the beginning of each CP experiment. After baseline measurements were obtained, saline and varying concentrations of NaOV were applied to the TM in all three CP experiments. Table 1 provides details such as the concentrations of NaOV administered during the experiments and the reason for termination of each CP experiment. The results can be grouped into one of two categories: 1) a substance-related change in CP produced by application of either saline or NaOV, or 2) a regular decrease in CP throughout the entire experiment.

3.1.1 Substance-related change in CP

The results of two CP experiments exhibit sustained changes in CP as a result of both saline and NaOV application. An example of a frequency-dependent response of a NaOV-related change in CP is shown in Figure 3.1 for Gerbil 040810. The solid black line is the baseline response taken prior to application of 10.0mM NaOV to the TM. The dotted black line is the first measurement taken post-10.0mM NaOV application. There

is a significant decrease in CP post-10.0mM NaOV application over the frequency range of 100 Hz to 8000 kHz.

The time-dependent response for Gerbil 040810 is shown in Figure 3.2. The baseline response and post-10.0mM NaOV response in Figure 3.1 corresponds with Trial 4 and Trial 5, respectively, in Figure 3.2. Before 10.0mM NaOV was applied to the TM, saline was administered to the TM between Trial 1 and Trial 2. At 1000 Hz, 2000 Hz, and 4000 Hz, saline application was associated with a 2-4 dB sustained increase in the CP response. The largest increase (4 dB) occurred at the highest frequency examined, 4000 Hz. 10.0mM NaOV was applied to the TM between Trial 4 and Trial 5. Associated with the application of NaOV is a sustained decrease in CP at 250 Hz, 500 Hz, 1000 Hz, 2000 Hz, and 4000 Hz. The decrease is smallest at 250 Hz (4 dB). The magnitude of the decrease increases with increasing frequency with the largest decrease of approximately 15 dB occurring at 4000 Hz. Saline was reapplied to the system between Trial 7 and Trial 8. There is no evidence that the second saline application washed-out or reversed the NaOV associated changes. In fact, there is a sizable saline-related decrease at 2000 Hz and 4000 Hz. There is, however, evidence of a saline-associated reversal at 500 Hz. The experiment was prematurely terminated by death of the animal after Trial 8.

Figure 3.3 shows the time-dependent response of Gerbil 040727. Saline was applied between Trial 0 and Trial 1 followed by applications of increasing concentrations of NaOV. Associated with saline application was a significant sustained increase in CP at 250 Hz, 500 Hz, and 1000 Hz and a sustained decrease in CP at 2000 Hz. The magnitude of the saline-associated changes was on the order of 2-5 dB. Between Trial 5 and Trial 6, 0.3mM NaOV was administered to the *pars tensa* of the TM; associated with

this application at 500 Hz and 1000 Hz was a 2.5 dB sustained increase. A 1.0mM NaOV application between Trial 9 and Trial 10 was associated with a 2 dB sustained decrease at 500 Hz and 1000 Hz. Application of 3.0mM NaOV and 10.0mM NaOV was not associated with a significant change in CP. Thus, while a single low-dose application of NaOV was associated with a sustained increase in CP, there is no evidence of a dose-dependent relationship between NaOV and CP.

3.1.2 A regular decrease in CP throughout the experiment

The results from one CP experiment, Gerbil 040726, are consistent with a gradual decrease in CP throughout the entire experiment. The frequency-dependent response for Gerbil 040726 is shown in Figure 3.4. The solid black line is the baseline response and the dotted black line is the last measurement taken during the experiment. There is a significant decrease in CP between the first measurement and the last measurement made. The decrease is consistent across the entire frequency range examined (i.e. 100 Hz to 8000 Hz). This decrease cannot be attributed to either saline application or NaOV application, but rather, is a gradual trend throughout the entire experiment.

The time-dependent CP response for Gerbil 040726 is shown in Figure 3.5. The baseline response and the last measurement in Figure 3.4 correspond to Trial 0 and Trial 12 in Figure 3.5. Saline was applied to the TM between Trail 1 and Trial 2. There is a transient decrease in CP of approximately 4 dB at 1000 Hz, 2000 Hz, and 4000 Hz post-saline application. Due to the gradual decrease in CP throughout the entire experiment as evidenced by the gentle slope downward from Trial 0 to Trial 12 in Figure 3.5, the health of the cochlea was questionable.

3.2 Laser Doppler Vibrometry (LDV) Results

The LDV results for the fourteen ears tested can be divided into four different experimental protocols. First, a series of saline-only measurements were performed in three ears. Second, saline and varying concentrations of NaOV were applied to the TM in nine ears. Third, in one ear only NEP was applied to the TM. Lastly, saline, NaOV, NEP, and CRB were administered to the TM in one ear. Table 2 provides details such as concentrations of each smooth muscle antagonist applied to the *pars tensa* of the TM and the reason for terminating each laser experiment. The magnitude of the LDV transfer function results, $|H| = |V_{\text{UMBO}} / P_{\text{EC}}|$, are classified into two categories: 1) no substance-related change in $|H|$, and 2) an upward or downward shift in the frequency dependence of $|H|$.

3.2.1 No substance-related change in $|H|$

The results of two saline-only experiments and three saline and NaOV experiments exhibit no substance-related change in $|H|$ when any substance was applied to the *pars tensa* of the TM. The frequency-dependent response for Gerbil 040707 is shown in Figure 3.6. The solid black line is the baseline condition and the dotted black line is 30 minutes post saline application. From 100 Hz to 8000 Hz, there is no significant change between the baseline condition and the post-saline condition.

Figure 3.7 shows the time-dependent response for Gerbil 040707. The baseline response and post-saline response in Figure 3.6 correspond to Trial 0 and Trial 14, respectively, in Figure 3.7. There are no significant changes in $|H|$ across the 14 trial experiment. The frequency dependent response curves and time-dependent response curves for Gerbil 040702_right, Gerbil 040811_right, Gerbil 040811_left, and Gerbil

040727 are very similar to those in Figure 3.6 and Figure 3.7. The name and concentration of the smooth muscle antagonist used in each experiment is detailed in Table 2.

In one ear only, Gerbil 040804, the middle ear space was opened and several applications of 10.0mM NaOV were applied to the mucosal side of the TM to see if a greater response could be induced. The frequency-dependent response for this special case is shown in Figure 3.8. The solid black line is the baseline response and the dotted black line is post-10.0mM NaOV application to the mucosal side of the TM. There is no significant change in $|H|$ between the two conditions from 100 Hz to 4000 Hz.

The time-dependent response for Gerbil 040804 is shown in Figure 3.9. The baseline condition and the post-10.0mM NaOV application in Figure 3.8 corresponds to Trial 1 and Trial 30, respectively, in Figure 3.9. The across trial magnitudes remained constant even after 4 applications of NaOV.

3.2.2 *Shift in the frequency dependence of $|H|$*

Eight ears exhibited a 2 dB or larger change in $|H|$ post-substance application. An example of a shift in the frequency-dependent response is a NaOV-related change in $|H|$ shown in Figure 3.10 for Gerbil 040625. The solid black line is pre-0.3mM NaOV application and the dotted black line is post-0.3mM NaOV application. There is a significant increase in $|H|$ from 100 Hz to 2500 Hz and there is a significant decrease in $|H|$ from 3200 Hz to 4500 Hz. At frequencies above 4500 Hz, the velocity measurement is too noisy to decipher the presence of a response. This shift in the frequency-dependent response is similar to that seen in six of the seven other ears that show a shift in $|H|$. Only the time-dependent response for these six ears will be discussed below. The one

frequency-dependent response that does not follow this trend will be discussed in detail later.

The time-dependent response for Gerbil 040625 is shown in Figure 3.11. The pre-0.3mM NaOV response and the post-0.3mM NaOV response in Figure 3.10 correspond to Trial 13 and Trial 17, respectively, in Figure 3.11. The experiment begins, however, with a set of seven baseline measurements. Trial 7 is the first measurement post-saline application. There is an 8dB transient decrease in $|H|$ at 250 Hz and an 8 dB sustained decrease at 500 Hz, 1000 Hz, and 2000 Hz between Trial 6 and Trial 8. 0.1mM NaOV was applied to the system between Trial 11 and Trial 12. At 4000 Hz, there is a 4 dB sustained increase between Trial 11 and Trial 12. The last drug applied during the experiment was 0.3mM NaOV, which was applied between Trial 15 and Trial 16. There was a significant sustained increase in $|H|$ at 250 Hz, 500 Hz, 1000 Hz, and 2000 Hz. The largest increase of approximately 12 dB occurred at the lowest frequency, 250 Hz, and the smallest increase (i.e. approximately 6 dB) occurred at 2000 Hz. At 4000 Hz, there was a small (i.e. 2 dB), but significant, sustained decrease in $|H|$.

Figure 3.12 shows the time-dependent response for Gerbil 040611. In this experiment, only saline was applied to the TM. Saline was introduced between Trial 5 and Trial 6. There is a sustained increase in $|H|$ of approximately 5 dB at 250 Hz and 500 Hz and, there is a 2 dB and 4 dB sustained decrease in $|H|$ at 2000 Hz and 4000 Hz, respectively. There is no change in $|H|$ at 1000 Hz.

The time-dependent response for Gerbil 040713 is shown in Figure 3.13. During post-saline, 0.1mM NaOV, 0.3mM NaOV, and 1.0mM NaOV application, there is no significant change in $|H|$. There are small spontaneous variations throughout the

experiment, but none of these transient changes are larger than 3dB or associated with substance application from Trial 0 to Trial 46. 3.0mM NaOV was applied between Trial 46 and Trial 47. At 500 Hz, there is a 3.5 dB transient increase in |H|. However, subsequent application of 10.0mM NaOV is not associated with a change in |H| at any of the frequencies examined. Thus, there appears to be no dose-dependent relationship between |H| and NaOV.

Figure 3.14 shows the time-dependent response for Gerbil 040810. The 250 Hz response and the 500 Hz response have been eliminated due to noise. The first two substances applied to the TM, saline and 10.0mM NaOV, elicited no change in |H| at 1000 Hz, 2000 Hz, and 4000 Hz. A second saline application, between Trial 25 and Trial 26, elicits a 6 dB transient increase at 1000 Hz and a 4 dB transient increase at 2000 Hz. A third saline application between Trial 34 and Trial 35 produces a 6 dB sustained increase at 1000 Hz and 2000 Hz. 30.0mM NaOV, the last substance applied to the TM between Trial 55 and Trial 56, was associated with a small 3 dB transient increase at 2000 Hz and a 3 dB sustained decrease at 4000 Hz.

Figure 3.15 shows the time-dependent response for Gerbil 040726. After saline application, there is no change across all five frequencies examined. 0.1mM NaOV was applied to the TM between Trial 11 and Trial 12, and there is a 3.5 dB sustained decrease in |H| at 4000 Hz. A second saline application was applied between Trial 20 and Trial 21. There was no significant changes in |H|. The umbo broke causing a tear in the TM at the end of the experiment, thus, it is unknown if the effect was reversible at 4000 Hz.

The last ear with a substance-associated shift in the frequency response used NaOV as the only smooth muscle antagonist applied to the TM. Figure 3.16 shows the

frequency-dependent response of Gerbil 040702_left, which is very different from the other frequency-dependent responses. The solid black line is the baseline response before any substance was added to the TM and the dotted black line is the response 15 minutes post-0.1mM NaOV application. There is a significant increase of approximately 6 dB in |H| between 3000 Hz and 5000 Hz. There are no other significant changes in |H| at any other frequency.

The time-dependent response for Gerbil 040702_left is shown in Figure 3.17. The baseline response and post-0.1mM NaOV response from Figure 3.16 corresponds to Trial 0 and Trial 18, respectively, in Figure 3.17. The 6 dB sustained increase seen in Figure 3.16 is evident between Trial 13 and Trial 25 in Figure 3.17 at 4000 Hz. There is a 4 dB decrease in |H| at 4000 Hz during 0.3mM NaOV application followed by a 3.5 dB sustained increase from Trial 24 to Trial 29. Application of 1.0mM NaOV between Trial 39 and Trial 40 builds upon this increase by approximately 6 dB. This 6 dB response is maintained throughout the rest of the experiment. The response pattern exhibits dose-dependent characteristics at 4000 Hz.

Figure 3.18 shows the time-dependent response for Gerbil 040819 before and after application of NEP. There was no change in |H| in response to saline application between Trial 2 and Trial 3. Saline application was followed by 4 applications of NEP. The first application of NEP was associated with a 5.5 dB transient increase at 250 Hz between Trial 10 and Trial 11. The second application of NEP, between Trial 13 and Trial 14, was associated with no change at any of the five frequencies examined. The third application of NEP was administered between Trial 18 and Trial 19. There was a 5.5 dB and 7.0 dB sustained increase at 250 Hz and 500 Hz, respectively. There was a 2

dB sustained increase at 1000 Hz, 2000 Hz, and 4000 Hz as well. The fourth application of saline, applied between Trial 21 and Trial 22 builds upon the previous increase by 3 dB at 250 Hz and 500 Hz. There was a 2 dB and 3 dB decrease in $|H|$, however, at 1000 Hz and 2000 Hz, respectively. There was no significant change at 4000 Hz due to the fourth application of 10.0mM NEP.

The last experiment, Gerbil 040818, is shown in Figure 3.19. The 250 Hz response has been eliminated due to low-frequency noise. In this experiment saline, 10.0mM NaOV, 10.0mM NEP, and 1.0mM CRB were applied to the TM over the course of the experiment. All four substances produced transient and sustained increases and decreases in $|H|$ across all four frequencies examined.

4. Discussion

4.1 Review of Results

4.1.1 Cochlear Potential

The CP results of this study suggest that 1) changes in CP can be induced by both saline and NaOV application, 2) saline application can either increase or decrease CP, 3) low concentrations of NaOV (i.e. $\leq 0.3\text{mM}$ NaOV) are associated with either an increase in CP or no change at all, 4) high concentrations of NaOV (i.e. $\geq 1.0\text{mM}$ NaOV) are associated with either a decrease in CP or no change at all and 5) there is no concentration-dependent relationship between CP and NaOV. These results should only be thought of as general trends, however, due to the small sample size ($n = 2$) used in the study.

4.1.2 Laser Doppler Vibrometry

The LDV results of this study suggest that 1) there is no saline or NaOV-induced change in $|H|$ in approximately 43% of all ears tested, 2) saline and/or smooth muscle antagonists are associated with a change in $|H|$ in approximately 57 % of all ears tested, 3) substance-related changes in $|H|$ show no dependence on frequency, 4) there is evidence in only one ear of a NaOV concentration-dependent change in $|H|$, and 5) with the exception of Gerbil 040818, the majority of observed drug-related shifts are increases in $|H|$. Given the variability associated with both saline and smooth muscle antagonist effects, we have no conclusive evidence of a drug-induced effect on umbo velocity,

4.1.3 No Response Laser Measurements

There was no substance-associated changes in $|H|$ in 43% of all ears tested. A possible explanation is failure for each substance to reach the TM during application. We used two different methods to apply the substances to the TM: syringe and pipette. Regardless of which method we used to apply the substances, at times it was very difficult to determine by microscope if the substance reached the surface of the TM. On several occasions, it appeared that the substance would cling to the bony ear canal due to surface tension. Thus, it is possible that the applied substance never reached the TM in these ears, and thus, no changes in $|H|$ would be observed.

4.1.4 Cochlear Potential Response versus $|H|$ response

There are three ears in this study in which both CP and $|H|$ were measured in the same ear. The time-dependent CP response of Gerbil 040810 (Figure 3.2) shows fewer trials than the time-dependent $|H|$ response (Figure 3.14) due to 1) there is one CP measurement for every three $|H|$ measurements (due to the non-linearity of the CP response as described in Section 2.5), and 2) CP was discontinued in this experiment because the stability of the cochlea was questionable post Trial 9, which corresponds to Trial 27 in the time-dependent $|H|$ response. Thus, only measurements taken pre-substance application, post-first saline application, and post-10.0mM NaOV application can be compared. There is a saline-associated increase (2-4 dB) and a 10.0mM NaOV-associated decrease (8-15 dB) in CP at 1000 Hz, 2000 Hz, and 4000 Hz. The laser measurements corresponding to saline and 10.0mM NaOV applications, however, show small (1-2 dB) saline-related *decreases* at 1000 Hz and 2000 Hz. Transient changes of ± 2 dB are also observed in the laser response after 10.0mM NaOV application at 1000 Hz

and 2000 Hz. Laser changes in $|H|$ are associated with second and third saline applications and with 30.0mM NaOV application late in the experiment. Thus, the substance-related changes in CP and $|H|$ are not correlated.

The time-dependent response of Gerbil 040727 (Figure 3.3) shows a 0.3mM NaOV-related increase and a 1.0mM NaOV-related decrease in CP at 500 Hz and 1000 Hz. Although the time-dependent $|H|$ response for Gerbil 040727 is not shown, it is very similar to the no change in $|H|$ response shown in Figure 3.7. Thus, the substance-associated changes in CP are not correlated with mechanical changes at the TM in Gerbil 040727.

CP and $|H|$ measurements were also obtained from Gerbil 040726. The time-dependent CP response (Figure 3.5) shows a gradual decrease in CP throughout the entire experiment that we chose not to classify as a substance-related response. In summary, while we have limited comparisons of substance-associated changes in CP and $|H|$, the evidence that exists shows no correlation between CP-associated and $|H|$ -associated changes.

4.2 Yang and Henson (2002) Comparison

Yang and Henson (2002) measured the effect of saline and NaOV on cochlear microphonic (CM) threshold. The CM threshold was defined as the lowest level at which an extracellular response can be measured from the cochlea relative to a 2.0 μ V noise floor. An increase in CM threshold is analogous to a decrease in absolute CP (the measurement used in this study) and a decrease in CM threshold is the same as an increase in absolute CP. Yang and Henson (2002) found that application of saline had no effect on CM threshold, whereas, application of NaOV had a concentration-dependent

effect on CM threshold. The results of my study, however, show that application of both saline and NaOV are associated with significant changes in CP. Furthermore, my results do not show a concentration-dependent relationship between CP and NaOV. For instance, a change in CP was observed in Gerbil 040727 post 0.3mM NaOV and 1.0mM NaOV application, but no change in CP was observed post 3.0mM NaOV and 10.0mM NaOV application.

Yang and Henson (2002) found that application of low concentrations of NaOV (i.e. 0.1mM) elicited either a small change or no change in CM threshold. The magnitude of this decrease was on the order of 1 dB. This is similar to the effect observed in this study. In Gerbil 040727, we observed no change in CP post 0.1mM NaOV application and a slight increase of 2 dB in CP post 0.3mM NaOV application. At concentrations greater than 1.0mM NaOV, however, Yang and Henson (2002) found there was an increase in CM threshold. This increase in CM threshold ranged from 3 dB to 9 dB, depending on the concentration of NaOV administered. The results of this study found that high concentration of NaOV (> 1.0mM NaOV) could induce a decrease in CP (Gerbil 040727 and Gerbil 040810) like the results in Yang and Henson (2002) or have no effect at all (Gerbil 040727). The maximum decrease in this study (15 dB) was associated with 10.0mM NaOV.

Yang and Henson found that changes in CM threshold were frequency-dependent with the largest changes occurring at the lowest frequency examined (2.16kHz) and the smallest changes occurring at the highest frequency examined (9.11kHz). This trend was observed in one ear in this study. A change in CP was observed only at frequencies less than 1000 Hz in Gerbil 040727, but no change in CP was observed at frequencies greater

than 2000 Hz but less than 4000 Hz. In Gerbil 040810, however, the largest change in CP occurred at 4000 Hz and the smallest change occurred at 250 Hz. Thus, the results from this study would suggest that there is no frequency dependence between CP and NaOV application.

Lastly, Yang and Henson (2002) observed NEP-induced increases in CM threshold. Because NEP is a catecholamine hormone, these results suggest that adrenergic receptors are present on the responding smooth muscle cells. In my study, however, transient and sustained increases in $|H|$ were induced by norepinephrine (Figure 3.18 and Figure 3.19) as well as by a cholinergic drug, CRB (Figure 3.19). These results are consistent with one of two explanations: 1) Application of a catecholamine hormone and a choline ester are associated with changes in $|H|$, thus, both adrenergic and cholinergic receptors may be present on the smooth muscle fibers, or 2) Applications of saline, NaOV, NEP, and CRB are associated with changes in $|H|$, and therefore, application of any substance alters the mechanical properties of the TM in some unknown way. Given the large amount of saline-associated changes in $|H|$, the latter explanation is likely. Due to the small sample size of NEP ($n=2$) and CRB ($n=1$) measurements in this study, these results should be considered as general trends in the data.

4.3 Are smooth muscle fibers responsible for regulating tension of the TM?

Studies suggest that the unique peripheral positioning of TM structural fibers, smooth muscle fibers, or myofibroblasts coupled with the presence of unmyelinated nerve fibers and vascular networks near the smooth muscle fibers make these fibers prime candidates to create and maintain tension of the tympanic membrane (Kuijpers et al, 1999; Henson and Henson, 2000; and Henson et al, 2005). This hypothesis is

supported by the results of Yang and Henson (2002). The results of my study, however, do not support this hypothesis.

Yang and Henson (2002) performed two control experiments to confirm that smooth muscle antagonist application was not affecting the function of other structures in the middle ear space. First, NaOV was applied to the round window membrane of the cochlea. The time course of the effects observed in this control experiment did not correlate with the time course of changes observed in the experiment where NaOV was applied to the TM. In addition, saline could not reverse the changes in CM threshold post-NaOV round window application. Second, NaOV was applied to the tensor tympani muscle. No change in CM threshold was observed during this control experiment. The results from both control experiments support the hypothesis that the smooth muscle antagonists only act on the smooth muscle fibers of the *pars tensa*. Thus, any changes observed in CM threshold were a direct result of mechanical changes at the TM. Yang and Henson (2002) reasoned that if the smooth muscle antagonists were contracting the smooth muscle fibers of the *pars tensa*, then the stiffness of the TM would increase. This increase in stiffness would cause a decrease in sound transmission to the inner ear, thus elevating CM thresholds. In a simple spring and mass-controlled system, the low frequency response is dominated by the stiffness of the spring. Therefore, changes in stiffness should have the largest effect at low frequencies. Yang and Henson (2002) observed the largest changes in CM threshold at the lowest frequency examined, 2.16 kHz.

The results of the present study, however, suggest that when CP measurements are compared with laser measurements from the same ear, changes in CP are not

correlated with changes in $|H|$. Most changes I observed in $|H|$ were increases in the transfer function magnitude. An increase in $|H|$ would indicate a decrease in stiffness of the TM, not an increase. Also, in this study, we often saw the largest substance-induced changes at the highest frequency examined (4000 Hz), with little or no change at frequencies equal to or less than 1000 Hz. This altered change in frequency-dependence is not consistent with a simple stiffness change. Lastly, CP and $|H|$ responses show irregular changes (mostly increases) as a result of saline application. This suggests that if changes are occurring at the level of the TM, the changes are not necessarily due to changes in the mechanical properties of the smooth muscle fibers of the *pars tensa*.

4.4 Alternative functions for the smooth muscle fibers of the pars tensa

4.4.1 Regulation and control of blood flow

Blood vessels are known to have concentric arrays of smooth muscle cells, which allow the blood vessels to stretch and constrict. This relationship between smooth muscle and blood vessels helps to regulate and control blood flow through the system. Yang and Henson (2002) argue that while this theory is plausible, TEM micrographs of the annulus fibrosis of the pars tensa in the gerbil show that concentric arrays of smooth muscle cells around blood vessels are rare. Furthermore, they claim that concentric arrays of smooth muscle cells around blood vessels are not advantageous for altering the tension of the TM. Henson and Henson (2005), however, describe the annulus fibrosis of the gerbil as having few blood vessels but an extensive network of small channels that have the ultrastructural appearance of lymphatic capillaries. Smooth muscle cells present at the entrance of capillaries help to regulate blood flow into each vessel by stretching and constricting the capillary entrance. However, if smooth muscle fibers regulate and

control capillary blood flow in the gerbil ear, it seems likely that changes these fibers produce in blood flow would be small enough that no effect on TM tension would be observed.

4.4.2 Pressure Sensation

In 1911, Wilson discovered modified Vater-Pacinian corpuscles in the periphery of the TM associated with capsulated nerve endings. Nagai and Tono (1989) found that the features of these corpuscles were similar to mechano-receptors. If these corpuscles serve as mechano-receptors, Nagai and Tono (1989) suggest their function might be to detect pressure changes in the middle ear cavity. For instance, if the tension of the TM is increased due to changes in middle ear pressure, the corpuscle might be “pressed upon” and a feeling of fullness in the ear would occur. Yang and Henson (2002) suggest that the corpuscles could provide feedback to the smooth muscle fibers to regulate muscle tone in the annulus fibrosis, and thus, help to alleviate the pressure change in the middle ear. However, minute pressure variations created by sound transduction through the middle ear do not cause a feeling of fullness in the normal ear. Large pressure variations create this sensation of fullness in the middle ear (i.e. such as the fullness a person experiences when increasing or decreasing in altitude on an airplane). If large pressure variations are needed to detect pressure changes in the middle ear cavity and thus activate a feedback control mechanism to the smooth muscle fibers of the annulus fibrosis, then the pressure variations in this study would be too small to do so. However, it might be interesting in the future to run a similar experiment as in this study while inducing large changes in static pressure within the middle ear cavity. The physiological range of such

pressures is ± 40 cm H₂O, which is much larger than the static pressure of <0.01 cm H₂O we used in this study.

4.4.3 Shape of the TM

Funnell and Laszlo (1974, 1975) introduced a finite-element model of the cat TM, which includes the conical shape of the eardrum, the *pars tensa*, the *pars flaccida*, the manubrium of the malleus, and the radial fibers of the *pars tensa*. They proposed that the radial fibers of the *pars tensa* alter the stiffness at the edge of the TM where it is attached to the ear canal wall. Thus, the TM can be modeled as a thin shell that has properties similar to a speaker cone. Quantitative analysis of this model (Funnell, 1977) suggests that the low frequency (< 2000 Hz) response characteristics of the TM are theoretically determined by the stiffness at the edge of the TM and the high frequency (> 2000 Hz) response characteristics of the TM are theoretically dependent on the characteristics on the center, or cone, of the TM. If the cone is relatively stiff, then the TM moves uniformly like a piston, whereas, if the cone is less stiff, the TM moves with several modes of vibration (supported by Decraemer et al., 1991).

Results obtained by Yang and Henson (2002) show the biggest effects of applying smooth muscle antagonists to the TM occur at the lowest frequency examined, 2.16 kHz. The results of my study, however, do not support the hypothesis of Yang and Henson (2002). The LDV results show that although large changes in $|H|$ can occur at low frequencies (250 Hz), large changes in $|H|$ most commonly occur at high frequencies (4000 Hz) during both saline and NaOV application. These results cannot be fully explained by a simple change in stiffness at the edge of the TM. The largest changes observed in $|H|$ at high frequencies in my study are consistent with a change in the

stiffness at the cone of the TM. A change in the stiffness of the cone could be associated with 1) contraction of the smooth muscle fibers of the annulus fibrosis (assuming that both saline and smooth muscle antagonists alter the properties of the smooth muscle fibers) or 2) application of any substance to the TM, which alters the shape of the TM in addition to changing the stiffness at the cone of the TM.

5. Conclusions

The primary goal of this thesis was to investigate the hypothesis of Yang and Henson (2002) that a smooth muscle system controls the tension of the *pars tensa*. We reasoned that the changes in TM tension that affect sound transmission have a primary effect on the mechanical motion of the *pars tensa* and the ossicles. A secondary goal of this thesis was to attempt to confirm the results obtained by Yang and Henson (2002).

The results of this study are highly variable and do not confirm the results of Yang and Henson (2002). Applications of saline, varying concentrations of NaOV, 10.0mM NEP, and 1.0mM CRB were associated with either 1) no change in CP or |H|, or 2) a substance-associated change in CP or |H|. When a substance-associated change in CP or |H| was present, the change in response could be increased or decreased, transient or sustained. Furthermore, in three experiments where both CP and |H| were measured in the same ear, there was no correlation found between the two measurements. These results suggest that any substance-associated changes in CP or |H| are not necessarily associated with contraction of smooth muscle fibers in the annulus fibrosis. These changes may be associated with other mechanical changes in the TM or in the middle ear space. In addition, changes observed in CP appear to be unrelated to changes in |H|. Therefore, whatever effect application of saline and smooth muscle antagonists have on the TM, these mechanical changes at the TM are independent of changes in cochlear function, and vice versa.

Yang and Henson (2002) observed the greatest drug-associated changes in CM threshold at 2.16kHz. As frequency increased, the drug-associated change decreased. These results are consistent with a change in stiffness in the periphery of the TM. Although we saw changes at the same frequencies examined in Yang and Henson (2002),

the trend was opposite of what Yang and Henson (2002) observed. That is, although we saw changes at all frequencies examined, the largest changes in CP and |H| usually occurred at the highest frequency (4000 Hz) examined. These results are not consistent with a simple change in stiffness in the periphery of the TM.

Our results suggest that application of various substances on the TM affect CP measurements in two of three ears and |H| measurements in eight of fourteen ears. However, the large effect of saline and the inconsistency of the observed effects do not support a simple drug-activated alteration in smooth muscle contractibility. Furthermore, the increases in TM velocity, the most common effect we observed, suggest a substance-induced decrease in stiffness rather than a stiffness increase suggested by Yang and Henson (2002).

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Table 1. Details of individual CP experiments. The left-most column lists the date that the gerbil was tested on. One gerbil was tested per day. Columns two through ten provide details on what substances were and were not applied to the tympanic membrane. The light gray columns correspond with concentrations of sodium orthovanadate applied to the TM, the medium gray column corresponds to the only concentration of NEP used in the study, 10.0mM, and the dark gray column corresponds to the only concentration of carbachol used in the study, 1.0mM. The two right-most columns detail the reason for terminating each experiment and if a shift in CP was associated with application of any substance used in the experiment. ----- Substance not applied to the TM. N- No drug associated change. Y-A drug associated change. Y/N-Mixed response or repeated application.

Gerbil	Saline	0.1mM	0.3mM	1.0mM	3.0mM	10.0mM	30.0mM	10.0mM	1.0mM	Termination of Experiment Response		
										TM Perforation	No	Equipment Failure
040726	N	N	-----	-----	-----	-----	-----	-----	-----	-----	No	
040727	Y	N	Y	Y	N	N	-----	-----	-----	-----	Yes	
040810	Y	-----	-----	-----	-----	Y	-----	-----	-----	-----	Yes	

Table 2. Details of individual laser experiments. The left-most column lists the date that the gerbil was tested on. One gerbil was tested per day. Columns two through ten provide details on what substances were and were not applied to the tympanic membrane. The light gray columns correspond with concentrations of sodium orthovanadate applied to the TM, the medium gray column corresponds to the only concentration of NEP used in the study, 10.0mM, and the dark gray column corresponds to the only concentration of carbachol used in the study, 1.0mM. The two right-most columns detail the reason for terminating each experiment and if a shift in [H] was associated with application of any substance used in the experiment. ----- Substance not applied to the TM. N- No drug associated change. Y-A drug associated change. Y/N-Mixed response or repeated application

Gerbil	Saline	0.1mM	0.3mM	1.0mM	3.0mM	10.0mM	30.0mM	10.0mM	1.0mM	Termination of Experiment Response	
										TM perforation	Yes
040611	Y	-----	-----	-----	-----	-----	-----	-----	-----	TM perforation	Yes
040625	Y	Y	Y	-----	-----	-----	-----	-----	-----	TM perforation	Yes
040702_right	N	N	N	-----	-----	-----	-----	-----	-----	TM perforation	No
040702_left	N	Y	Y	Y	-----	-----	-----	-----	-----	TM perforation	Yes
040707	N	-----	-----	-----	-----	-----	-----	-----	-----	TM perforation	No
040713	N	N	N	N	Y	N	-----	-----	-----	Animal expired	Yes
040726	N	Y	-----	-----	-----	-----	-----	-----	-----	TM perforation	Yes
040727	N	N	N	N	N	N	-----	-----	-----	Equipment Failure	No
040804	-----	-----	-----	-----	-----	N	-----	-----	-----	Data acquisition completed	No

040810	Y/N	----	----	----	----	----	----	N	Y	---	Animal expired	Yes
040811_right	N	----	----	----	----	----	----	----	---	---	TM perforation	No
040811_left	N	----	----	----	----	----	----	----	N	---	Data acquisition completed	No
040818	Y	----	----	----	----	----	----	Y	---	---	Animal expired	Yes
040819	N	----	----	----	----	----	----	----	---	Y/N	Animal expired	Yes

Figure 1.1

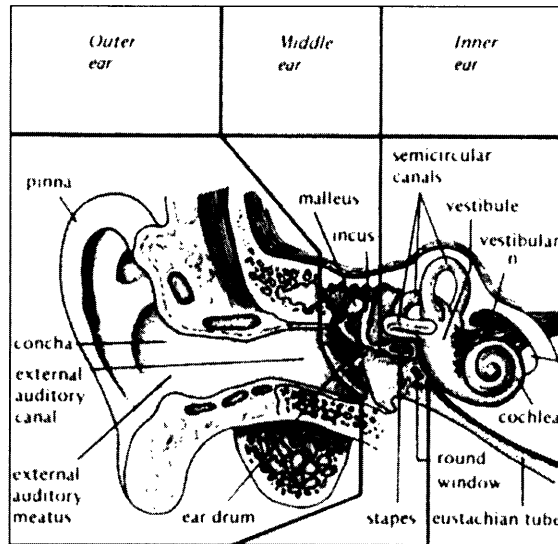


Figure 1.1. Cross-section of the human ear. The human ear is grossly divided into three sections: the outer ear, the middle ear, and the inner ear. The ear drum is commonly referred to as the tympanic membrane (TM). The drawing was adapted from Dallos (1973).

Figure 1.2

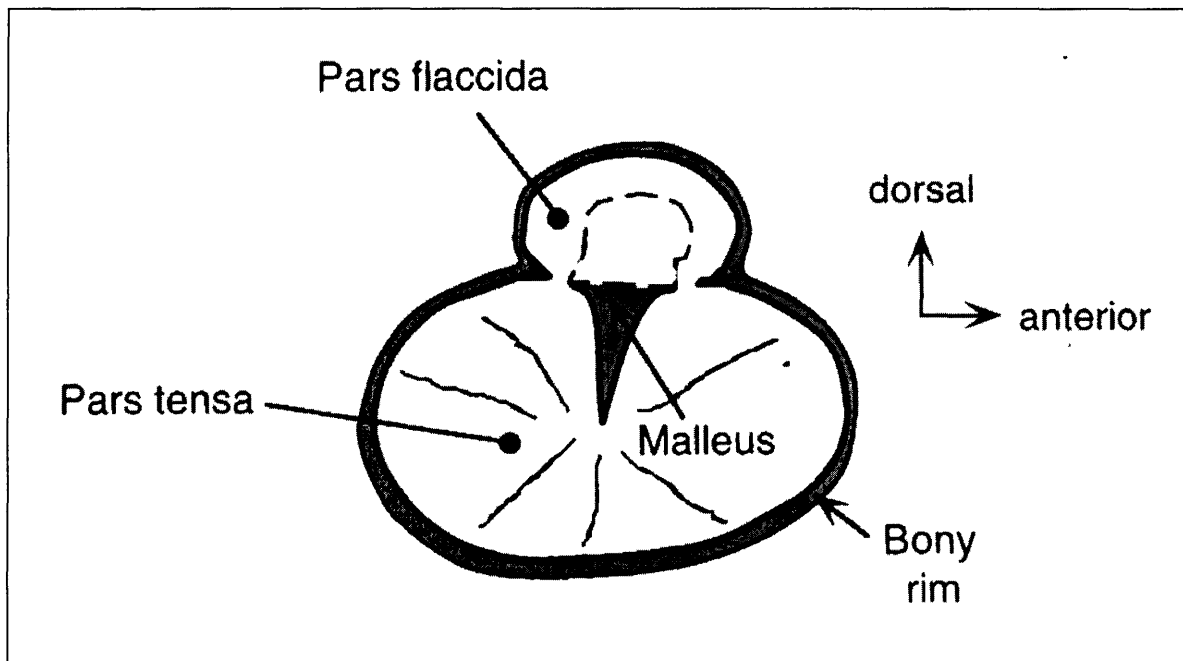


Figure 1.2. View of the gerbil tympanic membrane from the ear canal. The smaller, superior portion of the tympanic membrane (TM) is called the *pars flaccida* and the larger, inferior portion of the TM is called the *pars tensa*. The cone-like structure in the middle of the *pars tensa* is the shadow of the malleus attached to the inner surface of the nearly transparent TM. The drawing was adapted from Ravicz (1990).

Figure 1.3

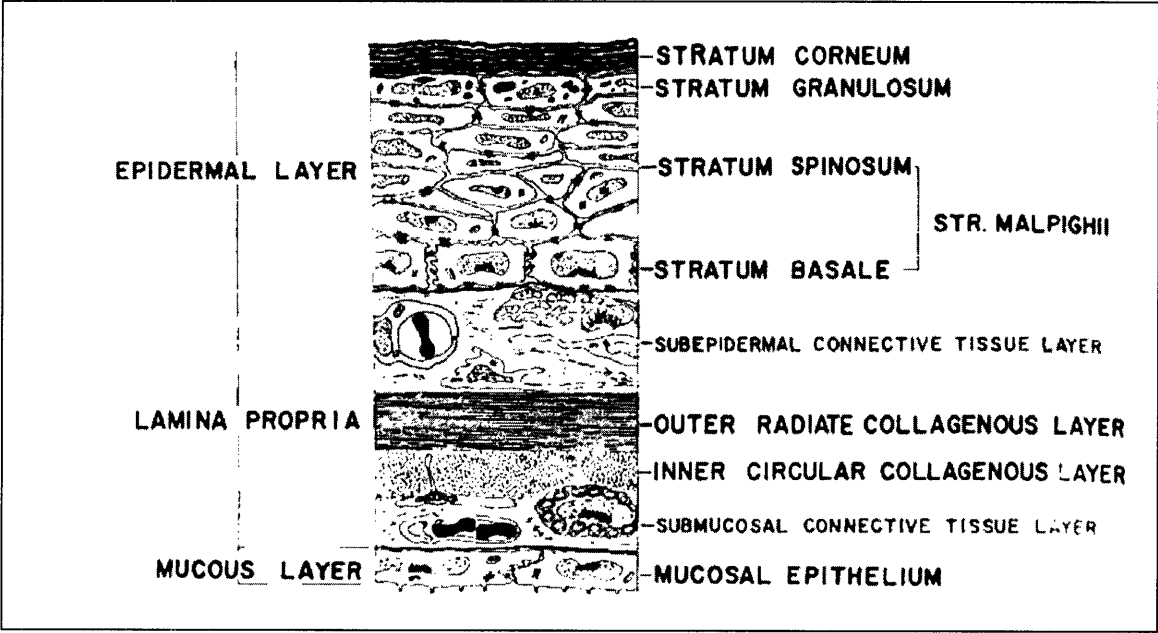


Figure 1.3. A cross-sectional view of the *pars tensa* of the tympanic membrane in the squirrel monkey. The drawing was adapted from Lim (1968a).

Figure 1.4

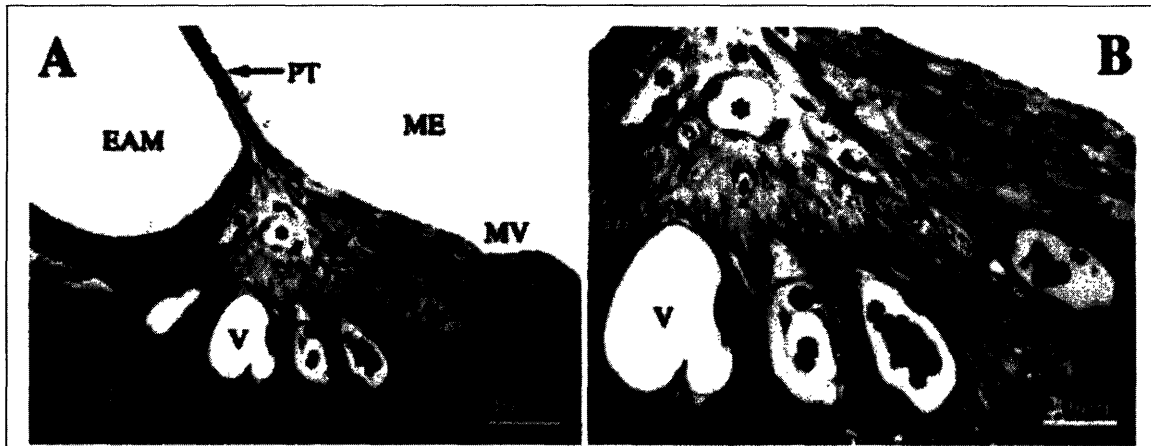


Figure 1.4. Light micrographs of the cross-sections of a tympanic bone and attached annulus fibrosis in the mustached bat. (A) Low magnification of the *pars tensa* (PT). The apical zone (AZ) of the annulus fibrosis consists of many small blood vessels (*) within a dense collagenous matrix. The myovascular zone (MV) of the annulus fibrosis consists of relatively large blood vessels (V) along with radially arranged muscle fibers. The middle ear space (ME), external auditory meatus (EAM), and tympanic bone (T) are also shown. (B) A higher magnification of the same section to provide a better orientation of the smooth muscle fibers (Henson and Henson, 2000).

Figure 1.5

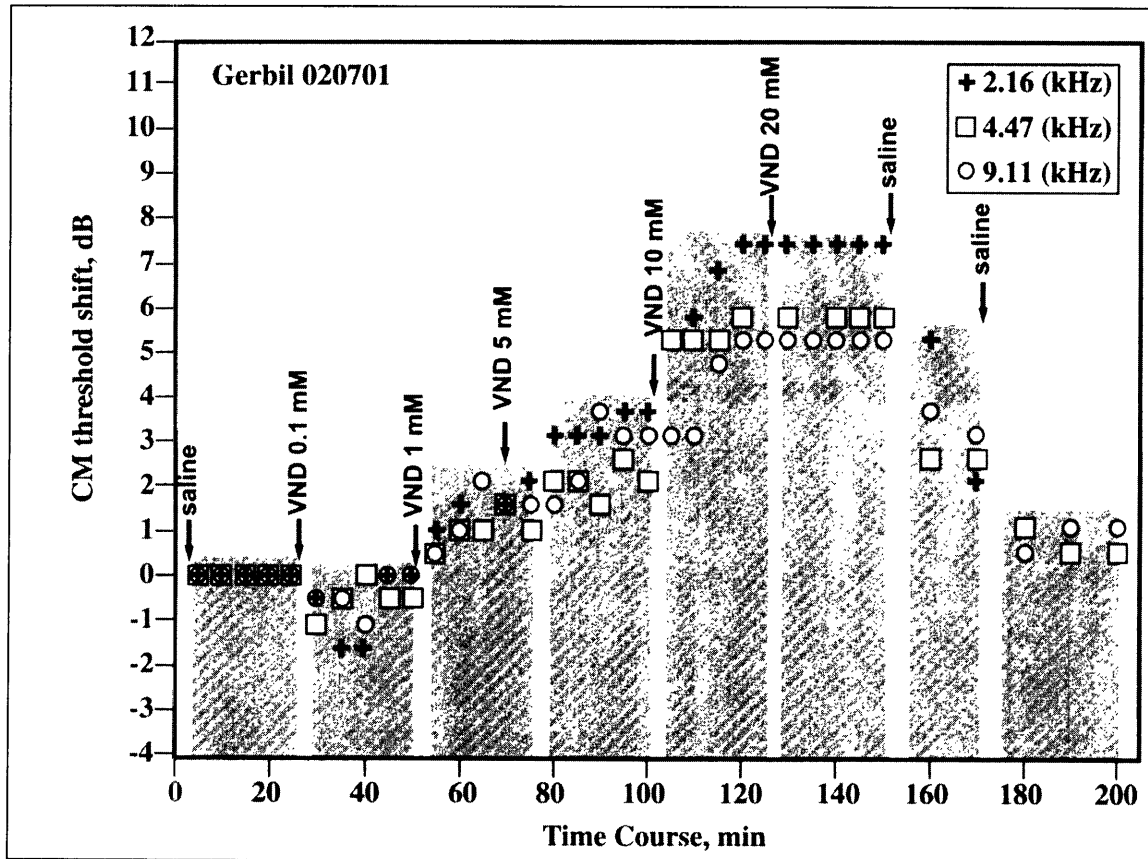


Figure 1.5. The effect of saline and varying concentrations of sodium orthovanadate (NaOV or VND) on the cochlear microphonic (CM) in one gerbil ear at 2.16 kHz, 4.47 kHz, and 9.11 kHz. Application of NaOV produced a dose-dependent increase in CM threshold. The largest change in CM threshold was observed at the lowest frequency examined, 2.16 kHz. The effect of NaOV on CM was reversible with saline washes (Yang and Henson, 2002).

Figure 1.6

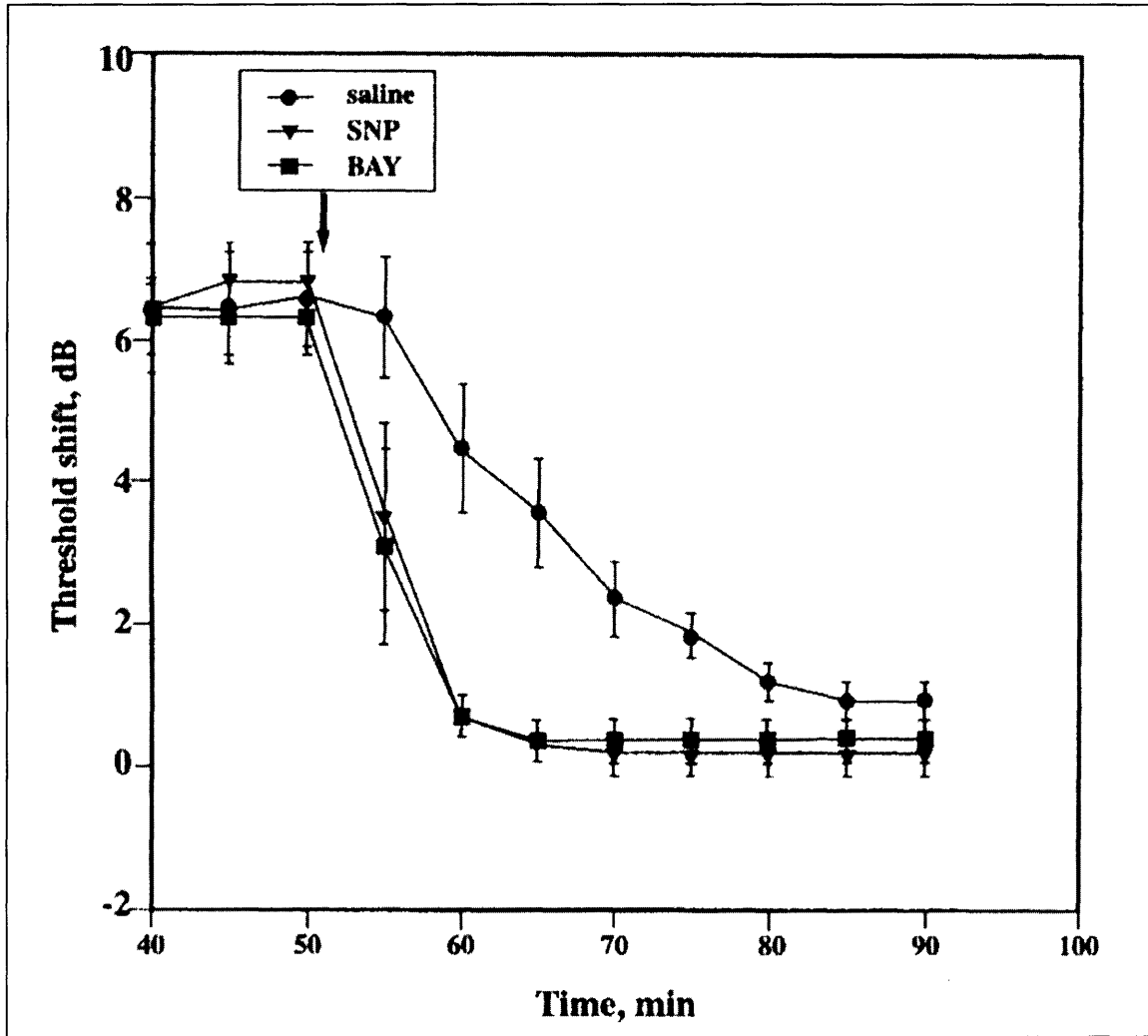


Figure 1.6. The effect of saline and smooth muscle relaxers on CM threshold. After activation with NaOV (not shown), the rate of recovery or washout was much faster when smooth muscle relaxers were used rather than saline (Yang and Henson, 2002).

Figure 1.7

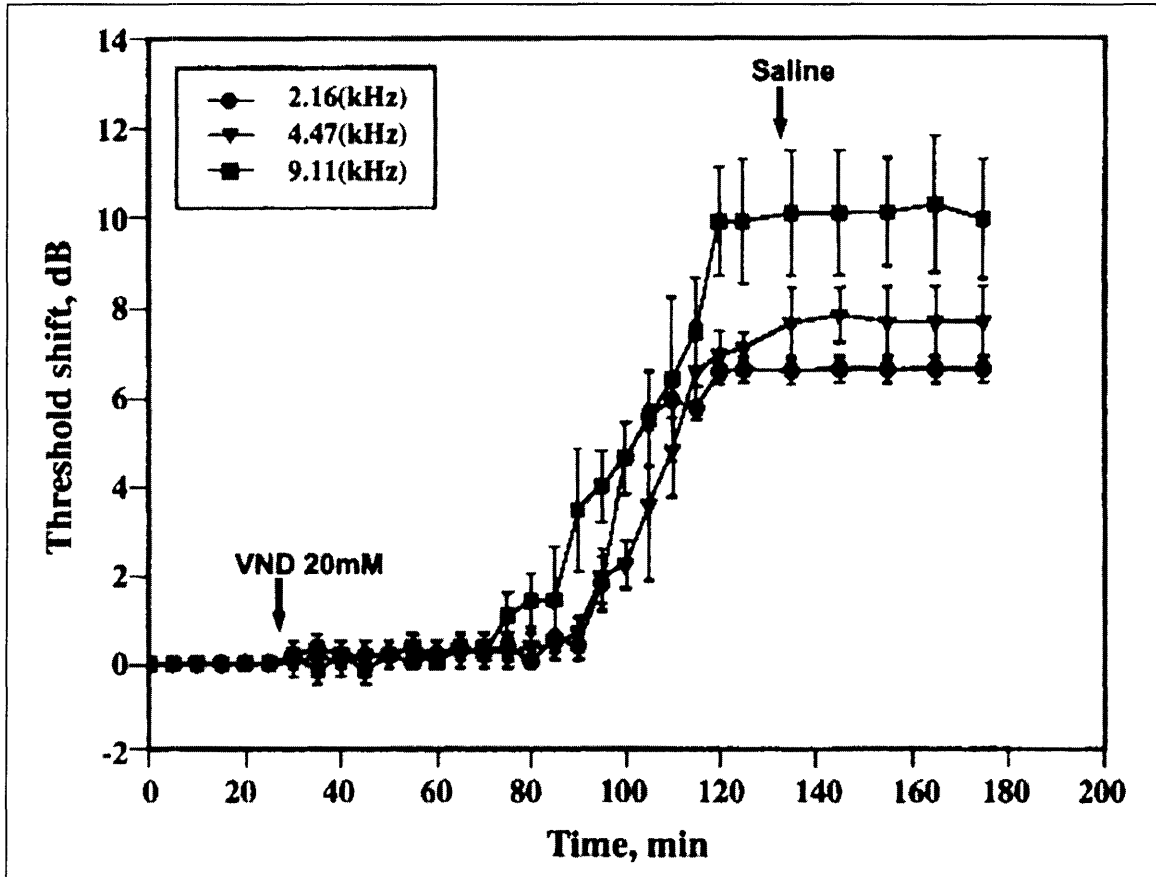


Figure 1.7. The effect of applying sodium orthovanadate (NaOV or VND) to the round window membrane of the gerbil ear. Application of NaOV 1) took longer to induce changes in CM threshold, 2) were largest at the highest frequency tested, and 3) could not be reversed with a saline wash. The results suggest that changes in CM threshold due to applications of drugs on the tympanic membrane are not due to the drug reaching the round window (Yang and Henson, 2002).

Figure 2.1

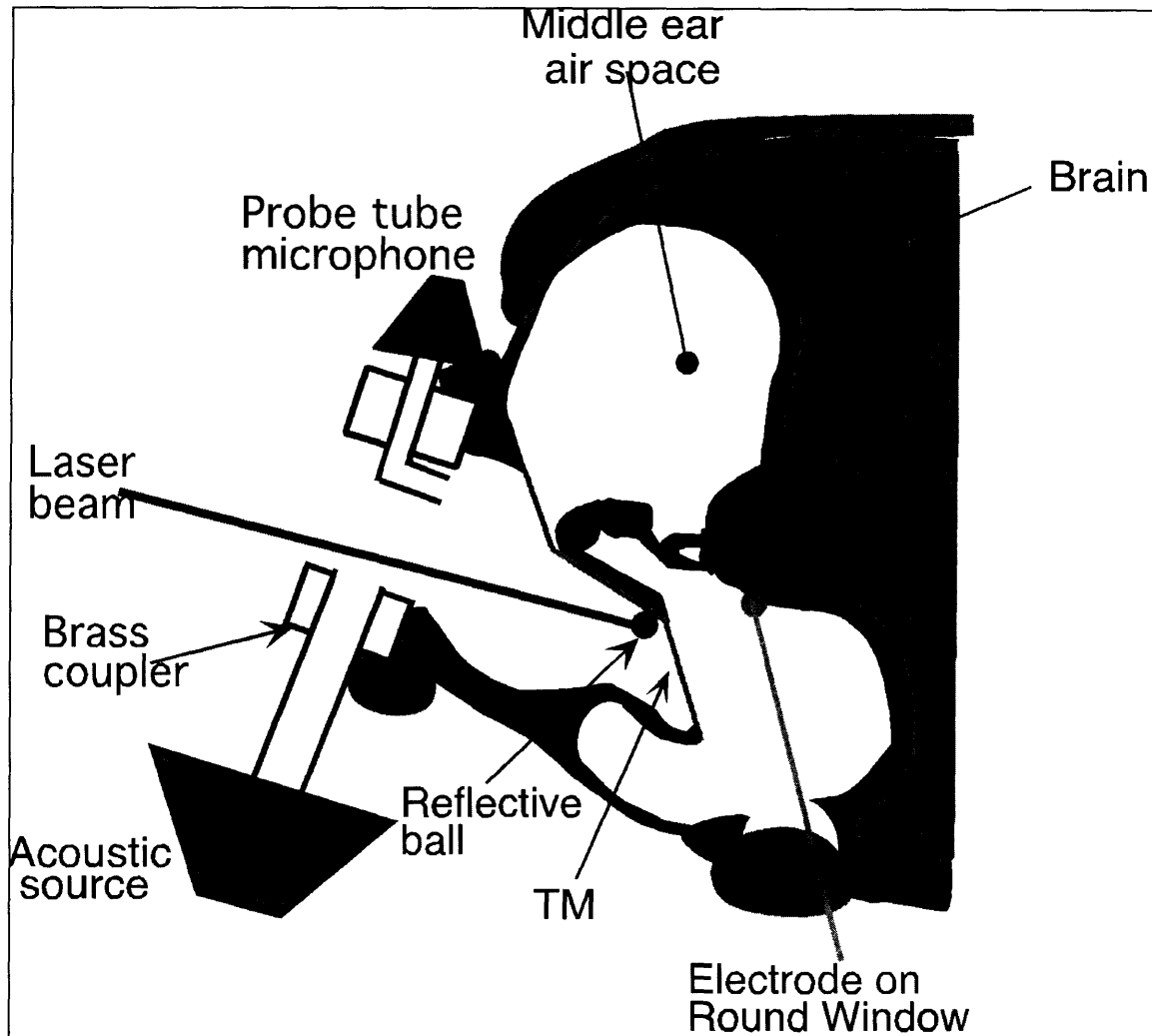


Figure 2.1. Experimental Setup for cochlear potential and laser measurements.

Figure 2.2

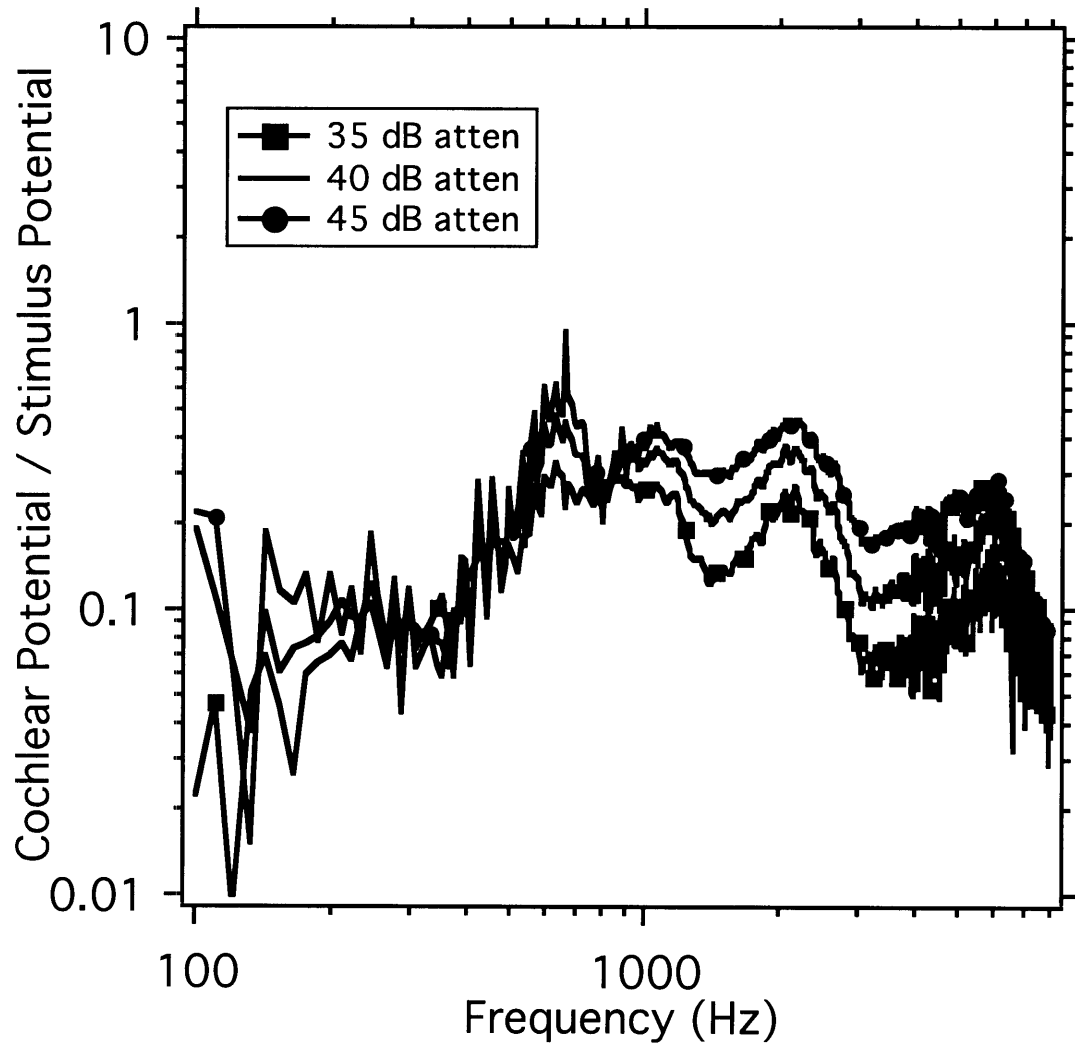


Figure 2.2. The frequency-dependent baseline CP response for Gerbil 040726 at three different attenuation levels: 35 dB, 40 dB, and 45 dB. Due to the non-linearity of the CP response, data analysis was performed at an attenuation level of 40 dB only. This level was low enough to prevent clipping of the CP response and high enough to reduce noise in the CP response.

Figure 2.3

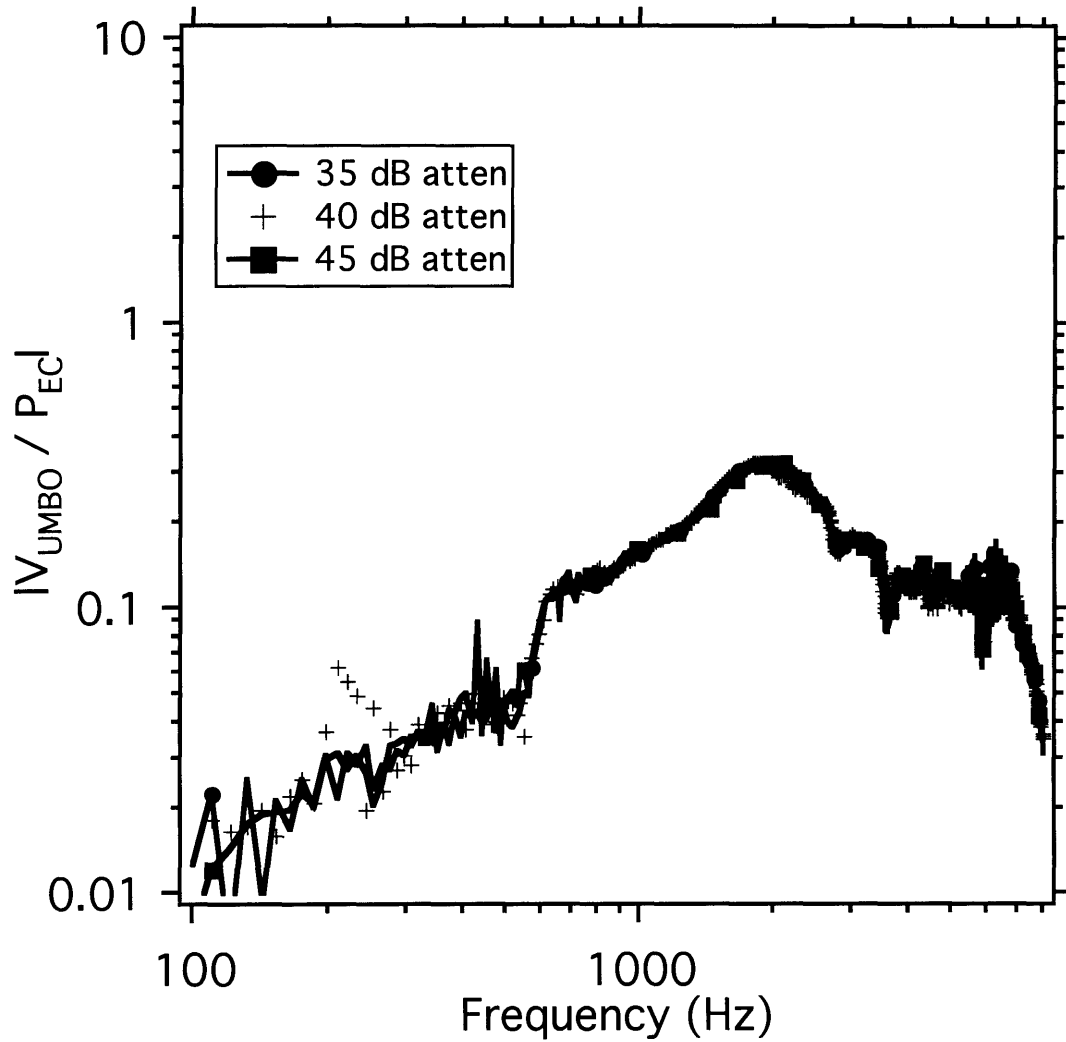


Figure 2.3. The frequency-dependent baseline laser response for Gerbil 040726 at three different attenuation levels: 35 dB, 40 dB, and 45 dB. The laser response is linear, and thus, all three attenuation levels were evaluated during data analysis.

Figure 3.1

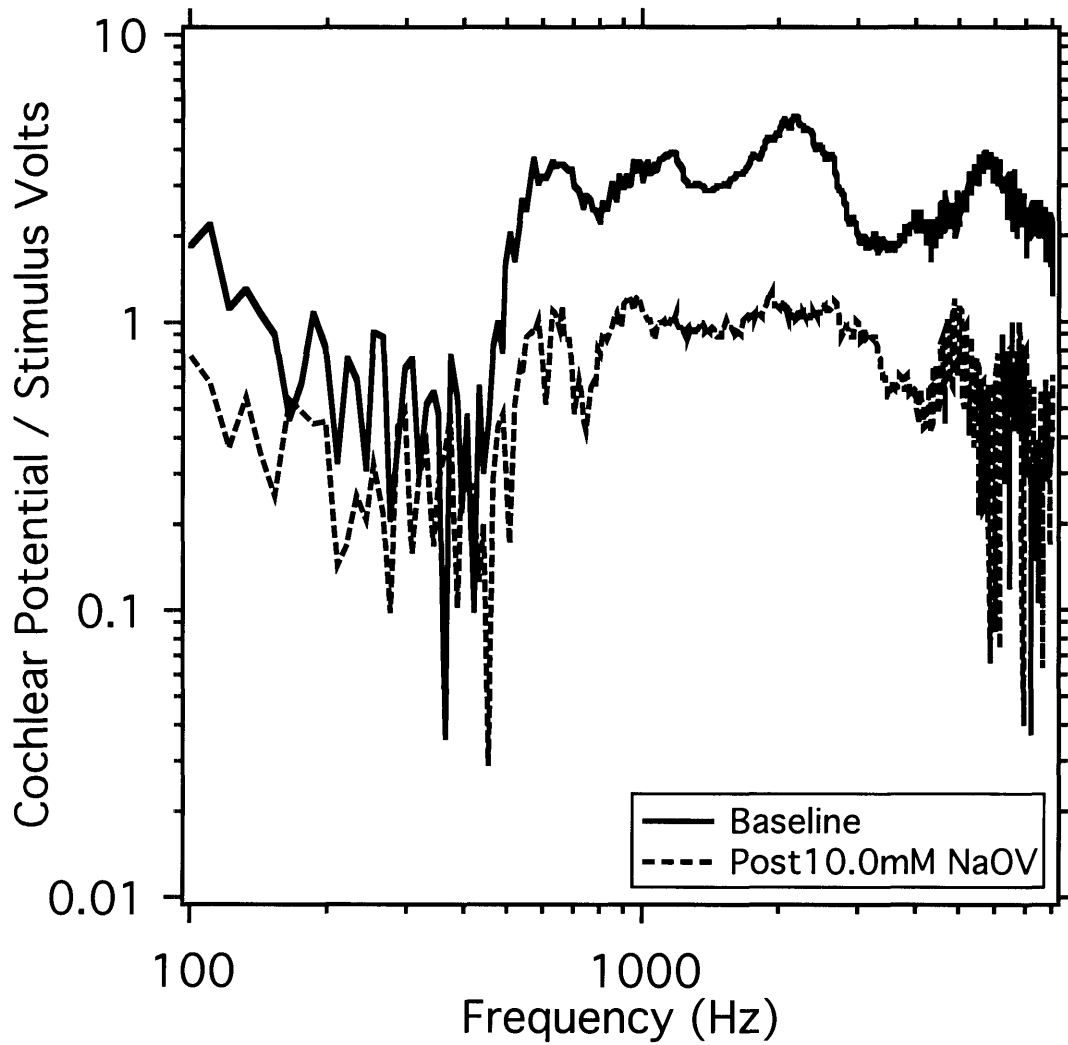


Figure 3.1. The frequency-dependent CP response for Gerbil 040810. The solid black line is the baseline response taken prior to 10.0mM NaOV application. The dotted black line is the first measurement taken post-10.0mM NaOV application. There is a significant decrease in CP post-10.0mM NaOV application from 100Hz to 8000 Hz.

Figure 3.2

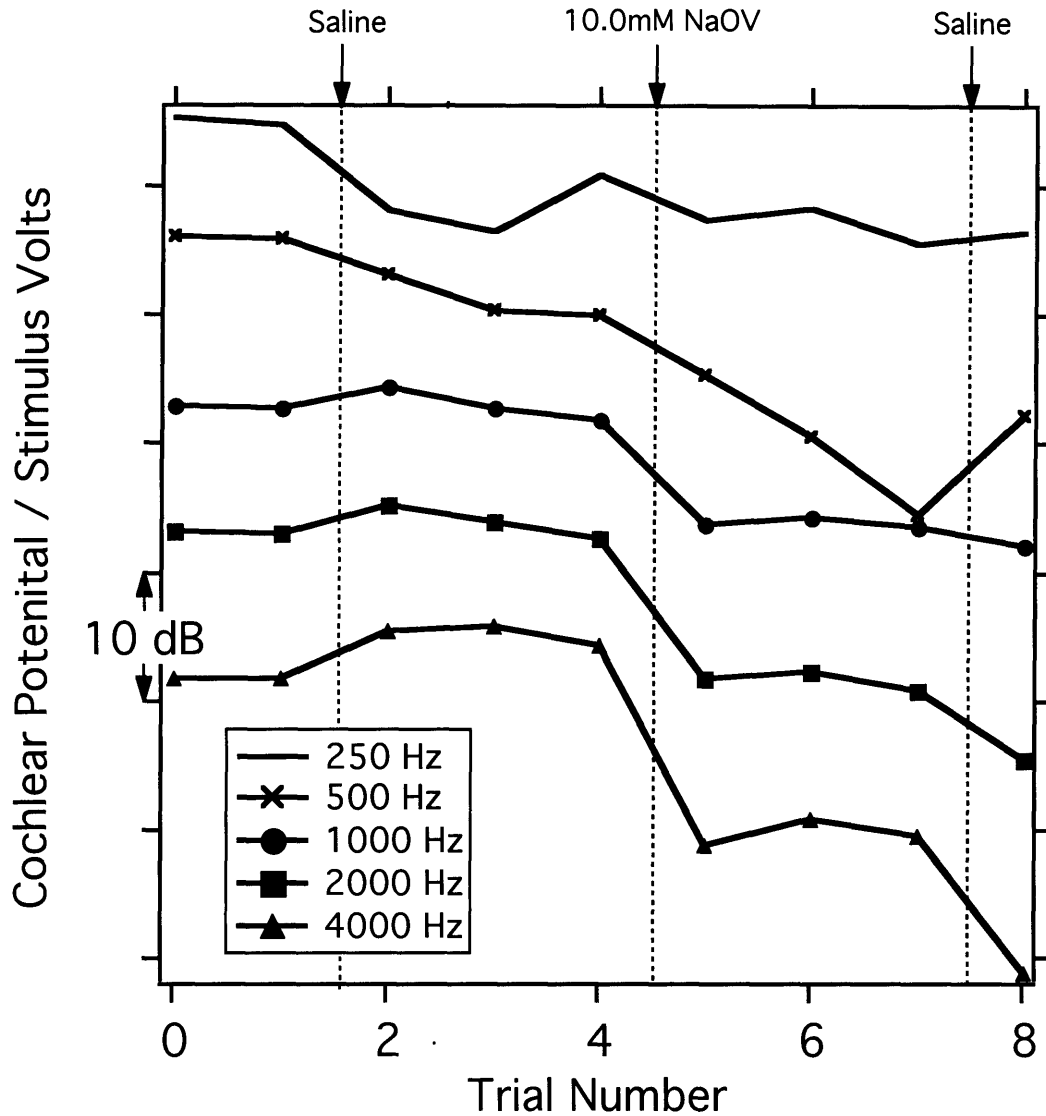


Figure 3.2. The time-dependent CP response for Gerbil 040810 at 250 Hz, 500 Hz, 1000 Hz, 2000 Hz, and 4000 Hz. Application of saline between Trial 1 and Trial 2 is associated with a change in CP at 1000 Hz, 2000 Hz, and 4000 Hz. Application of 10.0mM NaOV between Trial 4 and Trial 5 is associated with a large change in CP at 500 Hz, 1000 Hz, 2000 Hz, and 4000 Hz. There was evidence of a saline-induced reversal at 500 Hz.

Figure 3.3

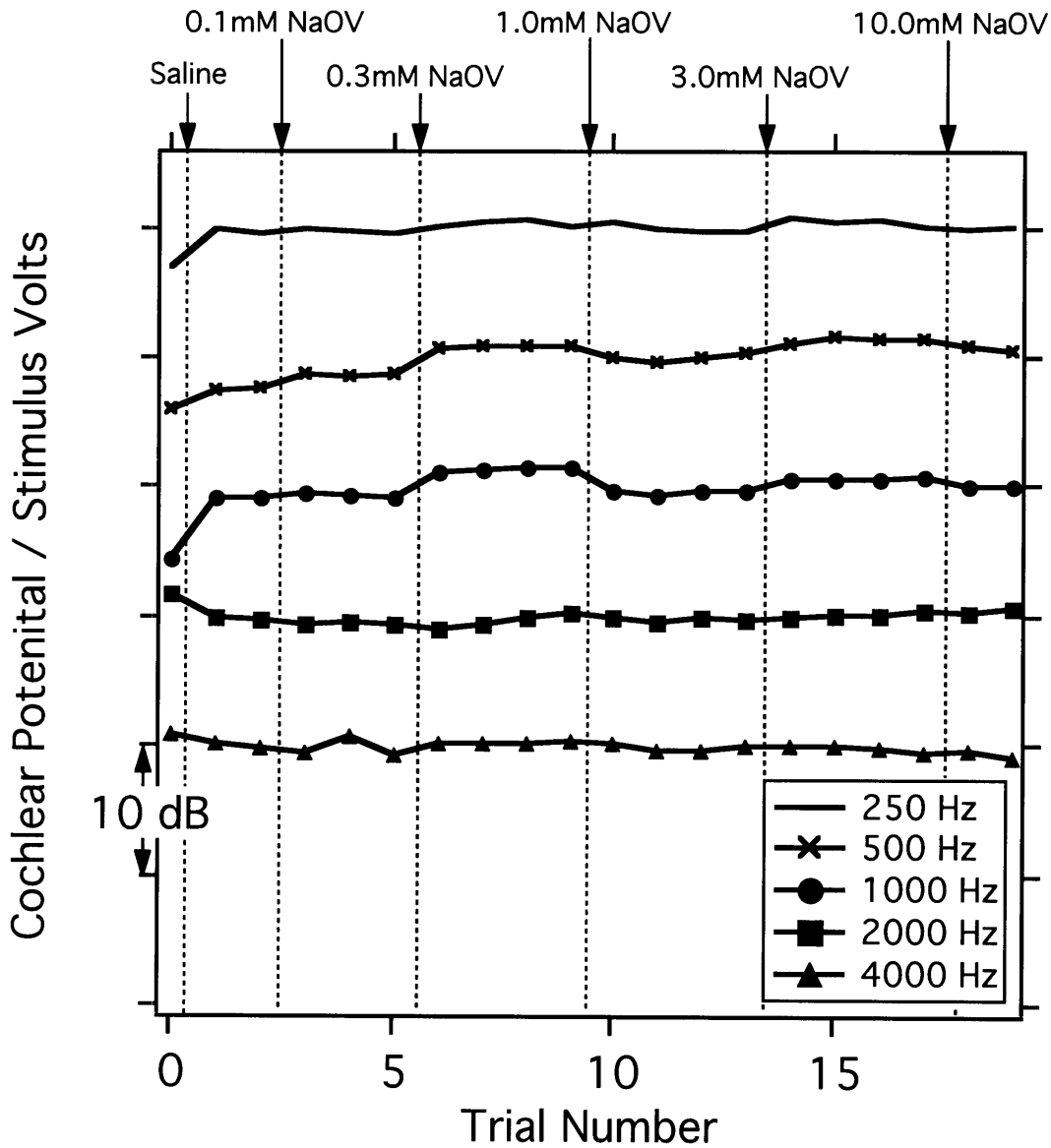


Figure 3.3. The time-dependent CP response for Gerbil 040727 at 250 Hz, 500 Hz, 1000 Hz, 2000 Hz, and 4000 Hz. Saline application was associated with an increase in CP at 250 Hz, 500 Hz, and 1000 Hz and a decrease at 2000 Hz. Application of 0.3mM NaOV was associated with an increase in CP at 500 Hz and 1000 Hz and application of 1.0mM NaOV was associated with a decrease in CP at 500 Hz and 1000 Hz. Higher concentrations of NaOV were not associated with a change in CP.

Figure 3.4

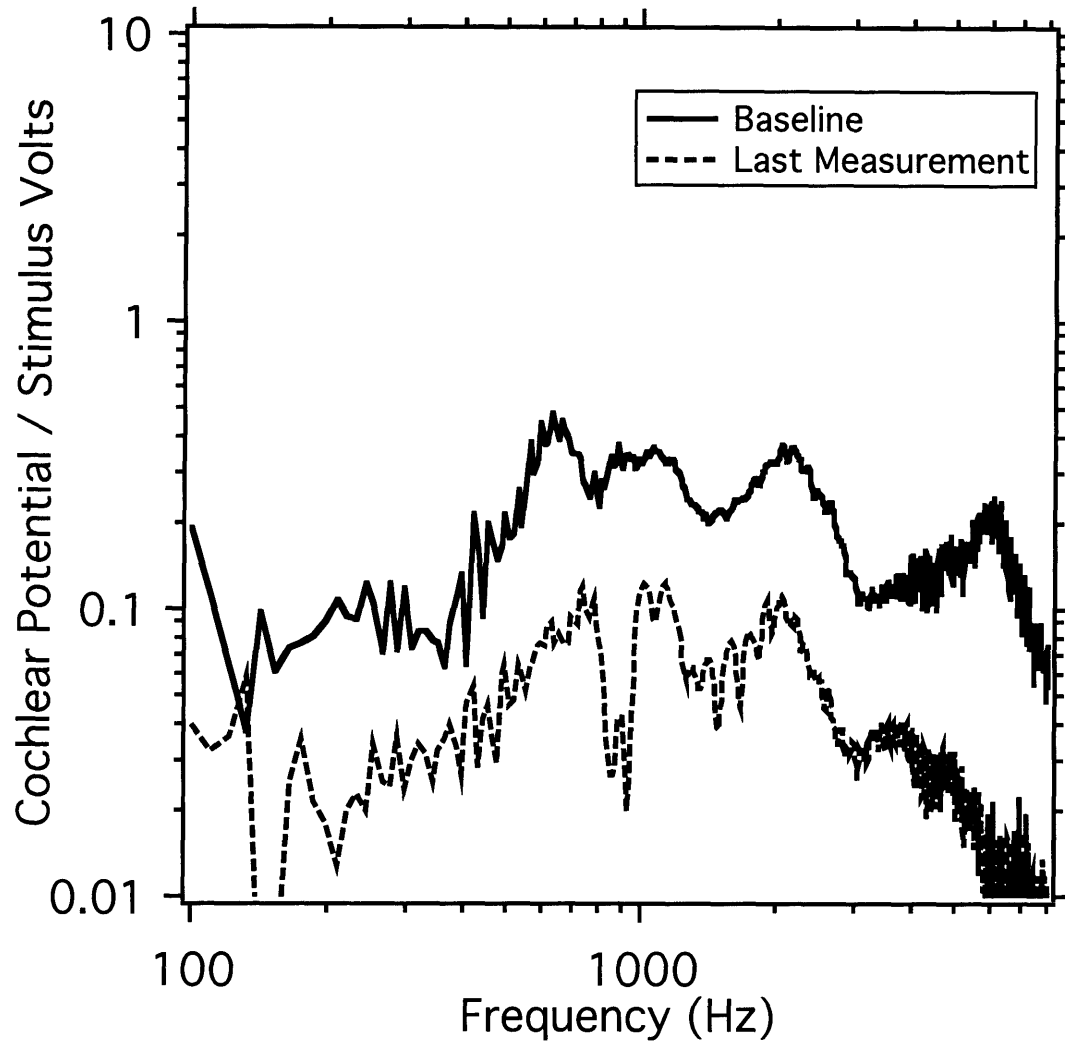


Figure 3.4. The frequency-dependent CP response for Gerbil 040726. The solid black line is a first (baseline) measurement and the dotted black line is the last measurement of the experiment. There is a large decrease in CP between the first measurement and the last measurement of the experiment from 100 Hz to 8000 Hz. This decrease in CP was a gradual trend throughout the entire experiment.

Figure 3.5

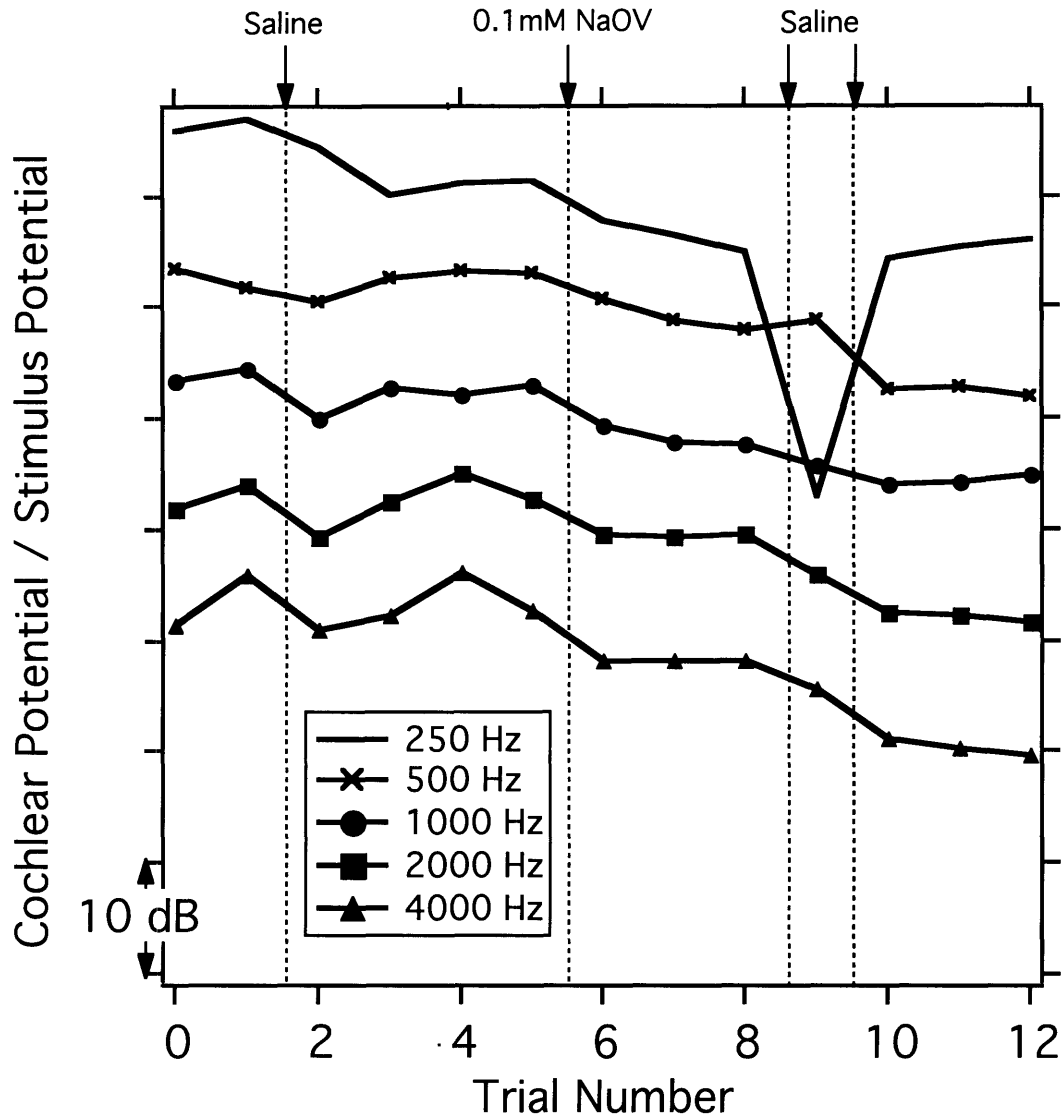


Figure 3.5. The time-dependent CP response for Gerbil 040726 at 250 Hz, 500 Hz, 1000 Hz, 2000 Hz, and 4000 Hz. There is a gradual decrease in CP from Trial 1 to Trial 12, which suggests that the health of the cochlea was questionable.

Figure 3.6

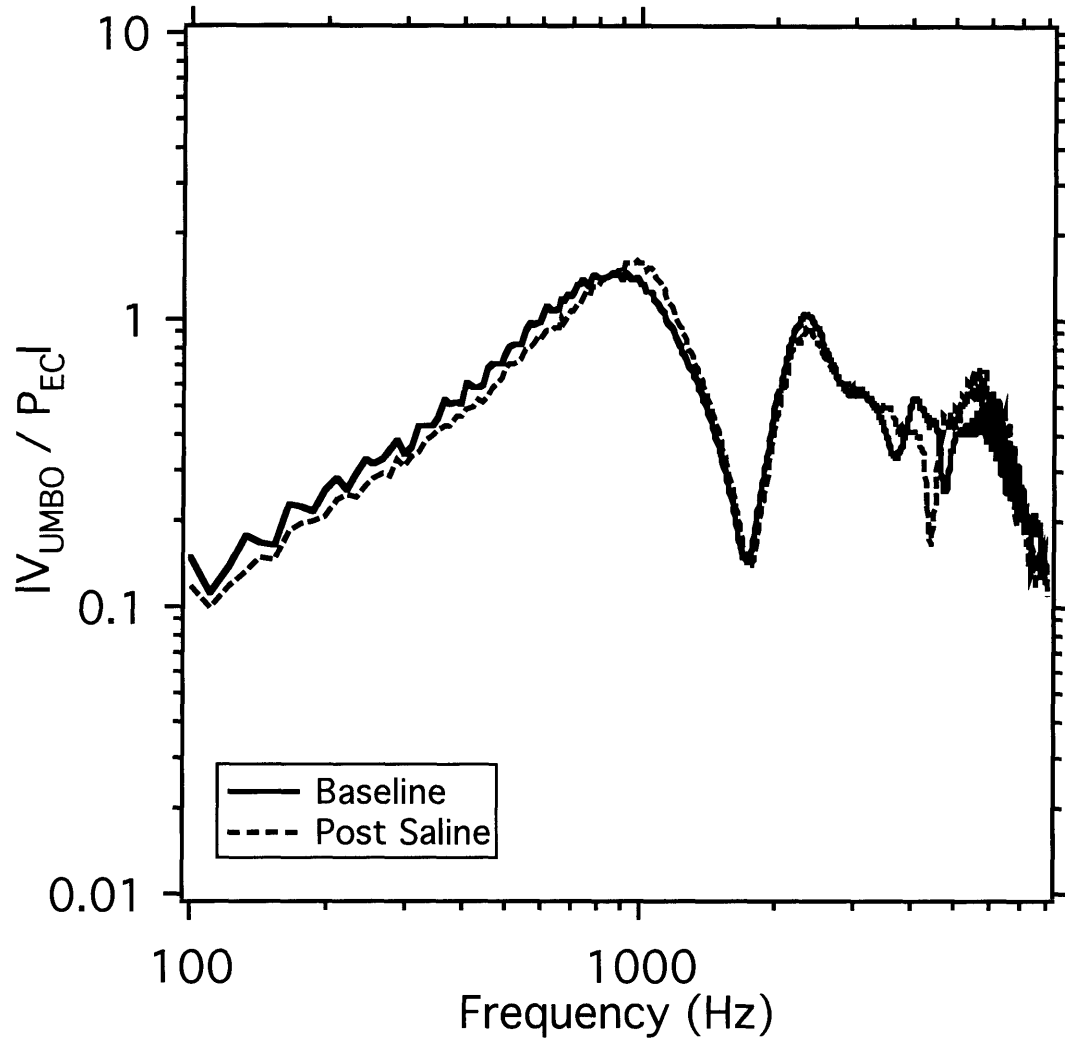


Figure 3.6. The frequency-dependent $|H|$ response for Gerbil 040707. The solid black line is the baseline condition and the dotted black line is 30 minutes post-saline application. There is no significant difference between the two transfer functions from 100 Hz, to 8000 Hz. This indicates saline application did not induce a change in $|H|$.

Figure 3.7

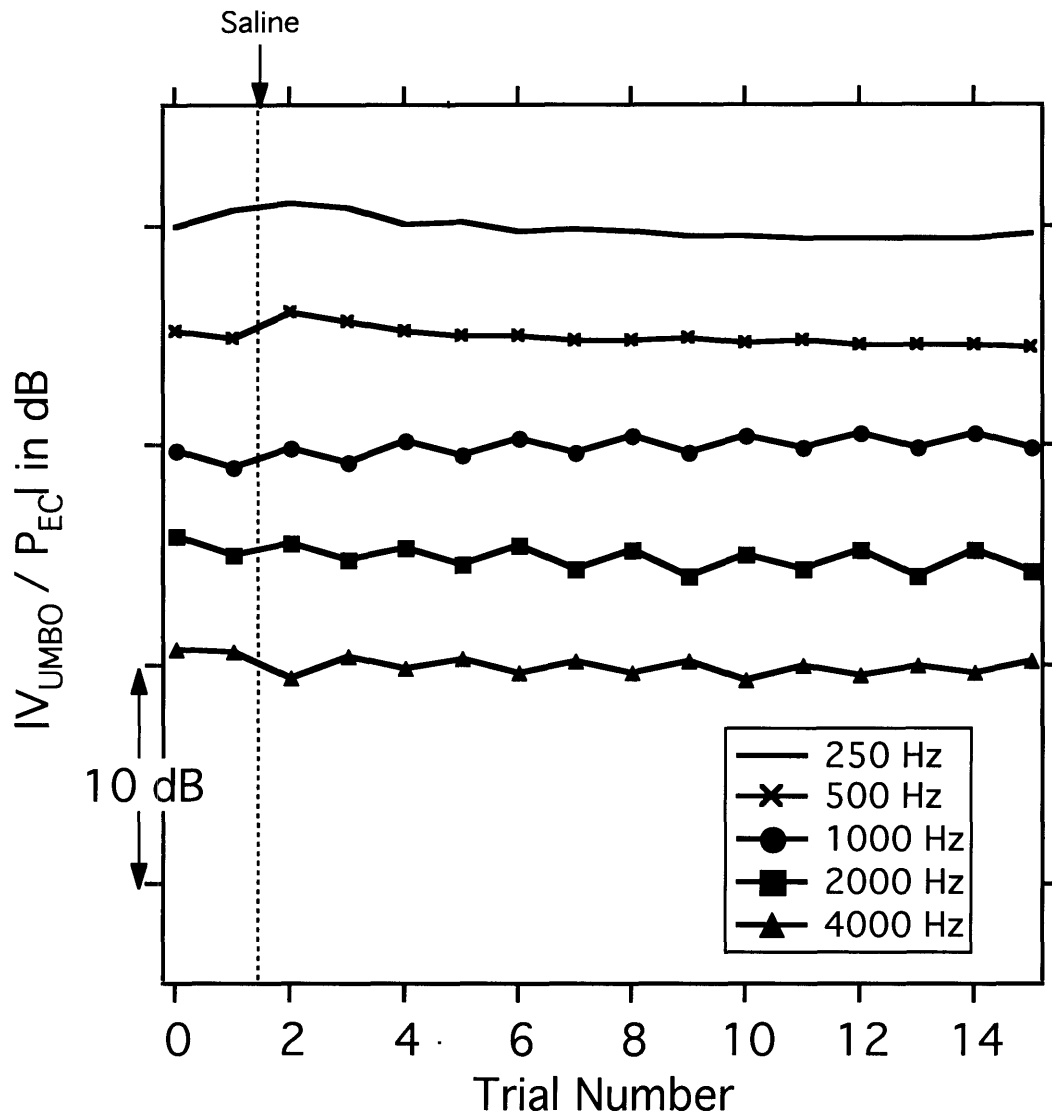


Figure 3.7. The time-dependent $[H]$ response for Gerbil 040707 at 250 Hz, 500 Hz, 1000 Hz, 2000 Hz, and 4000 Hz. There are no significant changes in $[H]$ across the entire experiment.

Figure 3.8

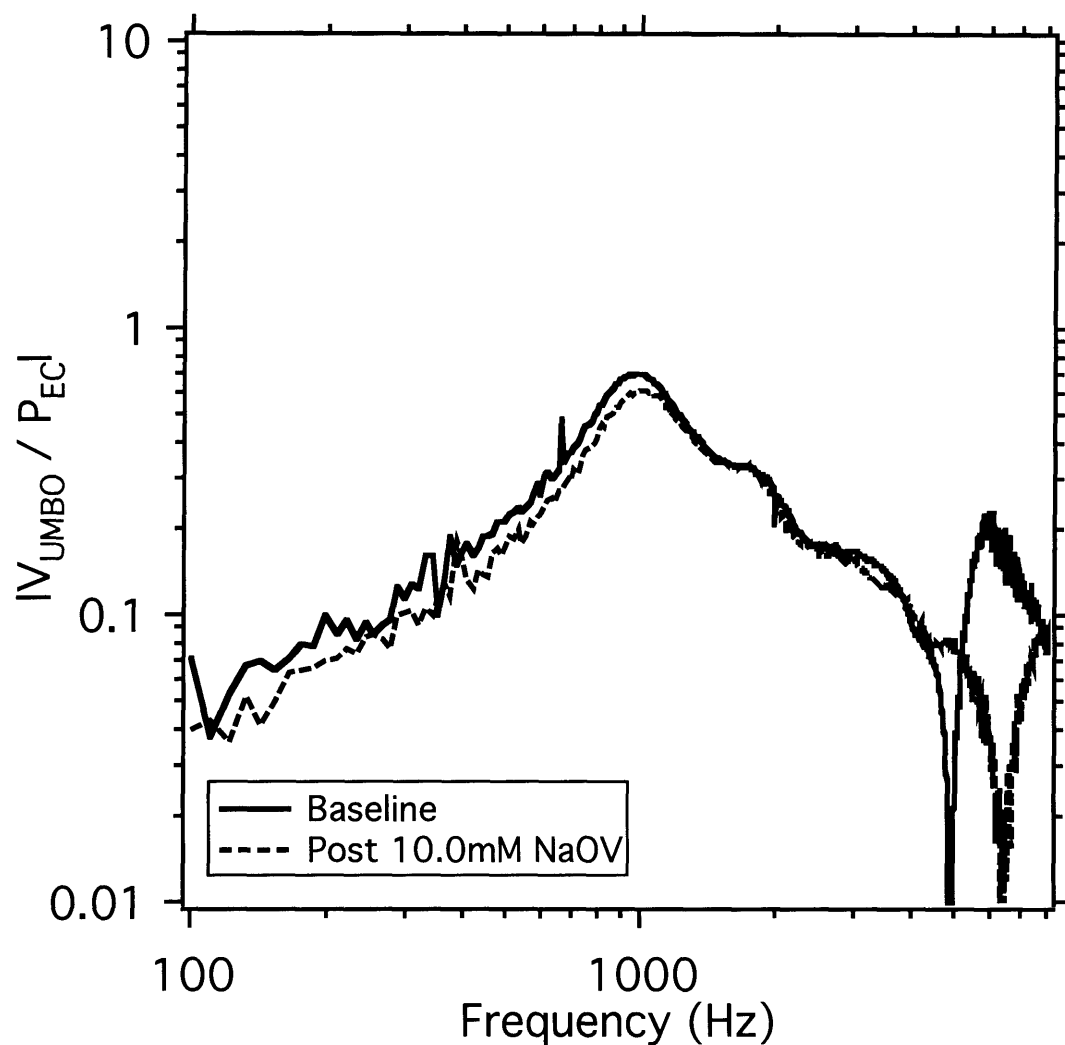


Figure 3.8. The frequency-dependent $|H|$ response for Gerbil 040804. The middle ear space was opened and 10.0mM NaOV was applied to the mucosal side of the TM. The solid black line is the baseline response and the dotted black line is the post-10.0mM NaOV response. There is no significant difference between the two transfer functions from 100 Hz to 4000 Hz.

Figure 3.9

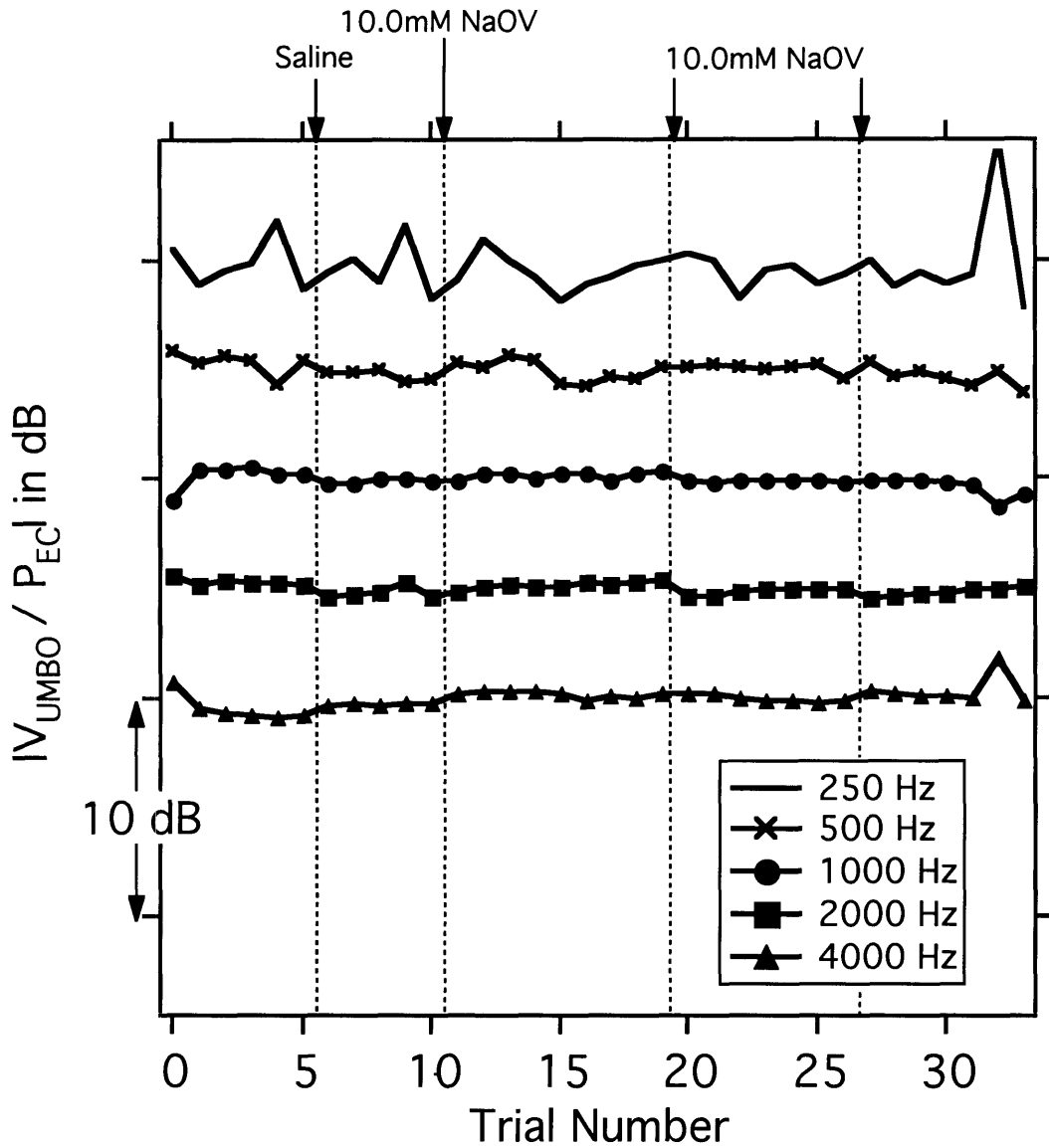


Figure 3.9. The time-dependent $|H|$ response for Gerbil 040804 at 250 Hz, 500 Hz, 1000 Hz, 2000 Hz, and 4000 Hz. The middle ear space was opened and 10.0mM NaOV was applied to the mucosal side of the TM. $|H|$ remains constant across the entire experiment for all five frequencies.

Figure 3.10

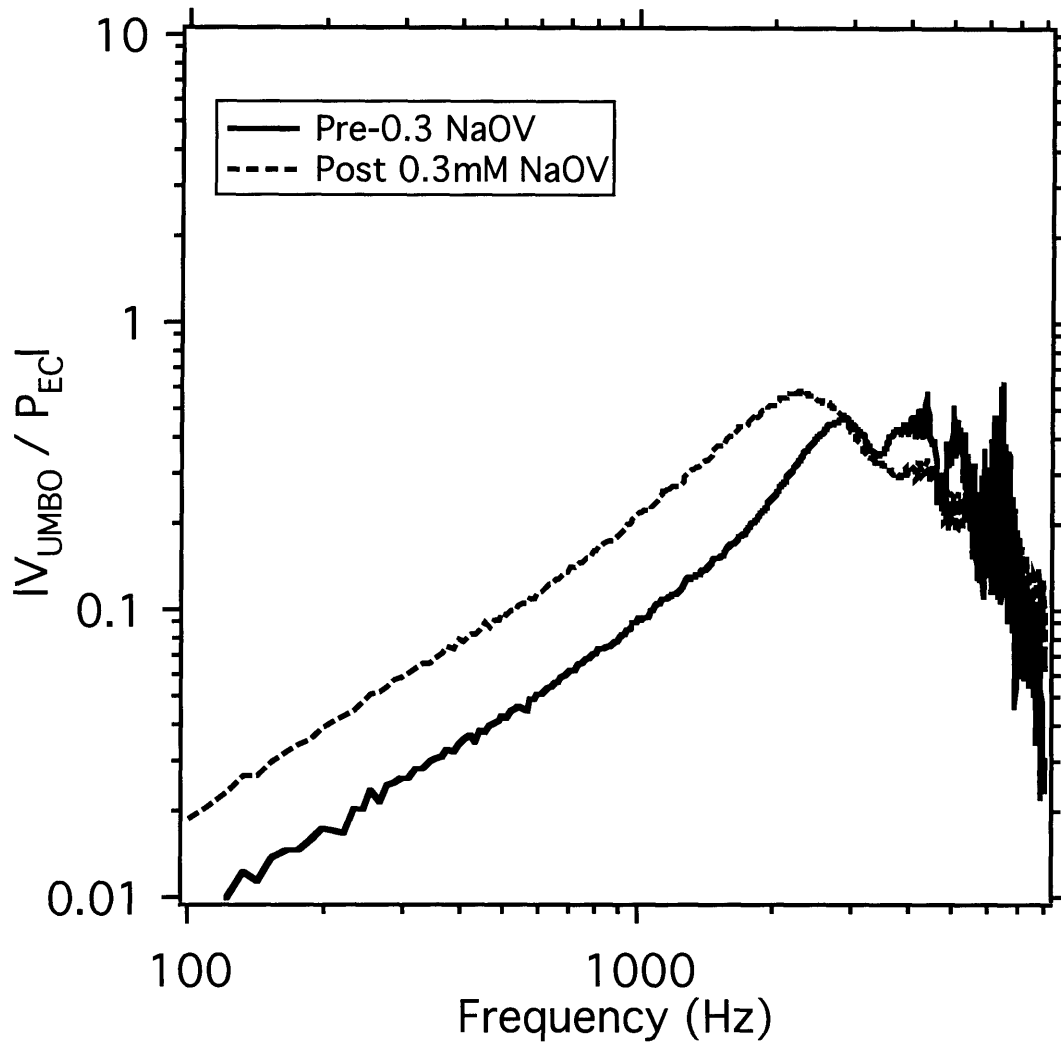


Figure 3.10. The frequency-dependent $|H|$ response for Gerbil 040625. The solid black line is a pre-0.3mM NaOV response and the dotted black line is a post-0.3mM NaOV response. There is a large increase in $|H|$ from 100 Hz to 2500 Hz and there is a large decrease in $|H|$ from 3200 Hz to 4500 Hz.

Figure 3.11

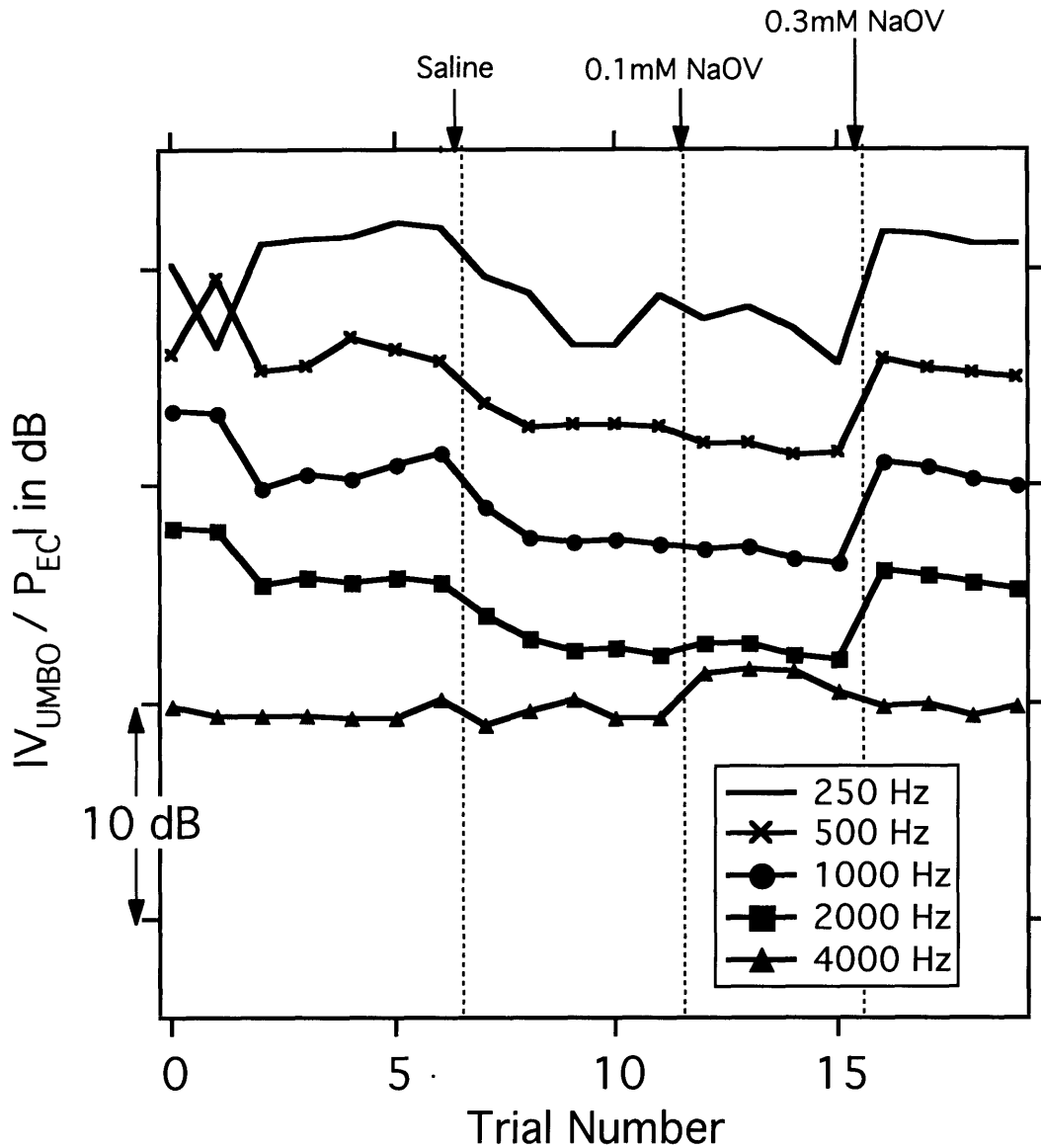


Figure 3.11. The time-dependent $|H|$ response for Gerbil 040625 at 250 Hz, 500 Hz, 1000 Hz, 2000 Hz, and 4000 Hz. Saline application was associated with a decrease in $|H|$ at 250 Hz, 500 Hz, 1000 Hz, and 2000 Hz. Application of 0.1mM NaOV was associated with a decrease in $|H|$ at 4000 Hz and application of 0.3mM NaOV was associated with large increase in $|H|$ at 250 Hz, 500 Hz, 1000 Hz, and 2000 Hz.

Figure 3.12

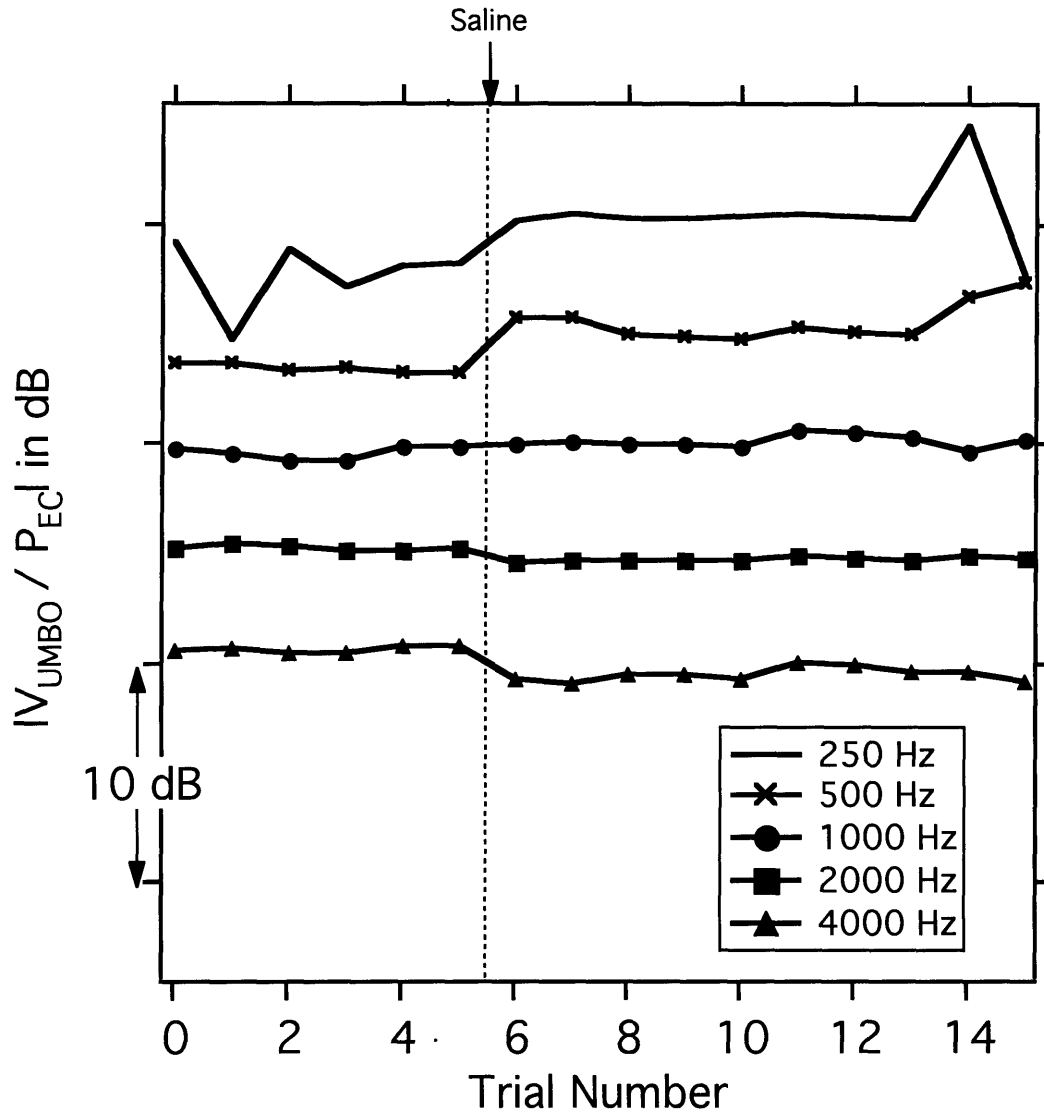


Figure 3.12. The time-dependent $|H|$ response for Gerbil 040611 at 250 Hz, 500 Hz, 1000 Hz, 2000 Hz, and 4000 Hz. Application of saline was associated with an increase in $|H|$ at 250 Hz and 500 Hz and a decrease in $|H|$ at 2000 Hz and 4000 Hz. There was no change in $|H|$ at 1000 Hz.

Figure 3.13

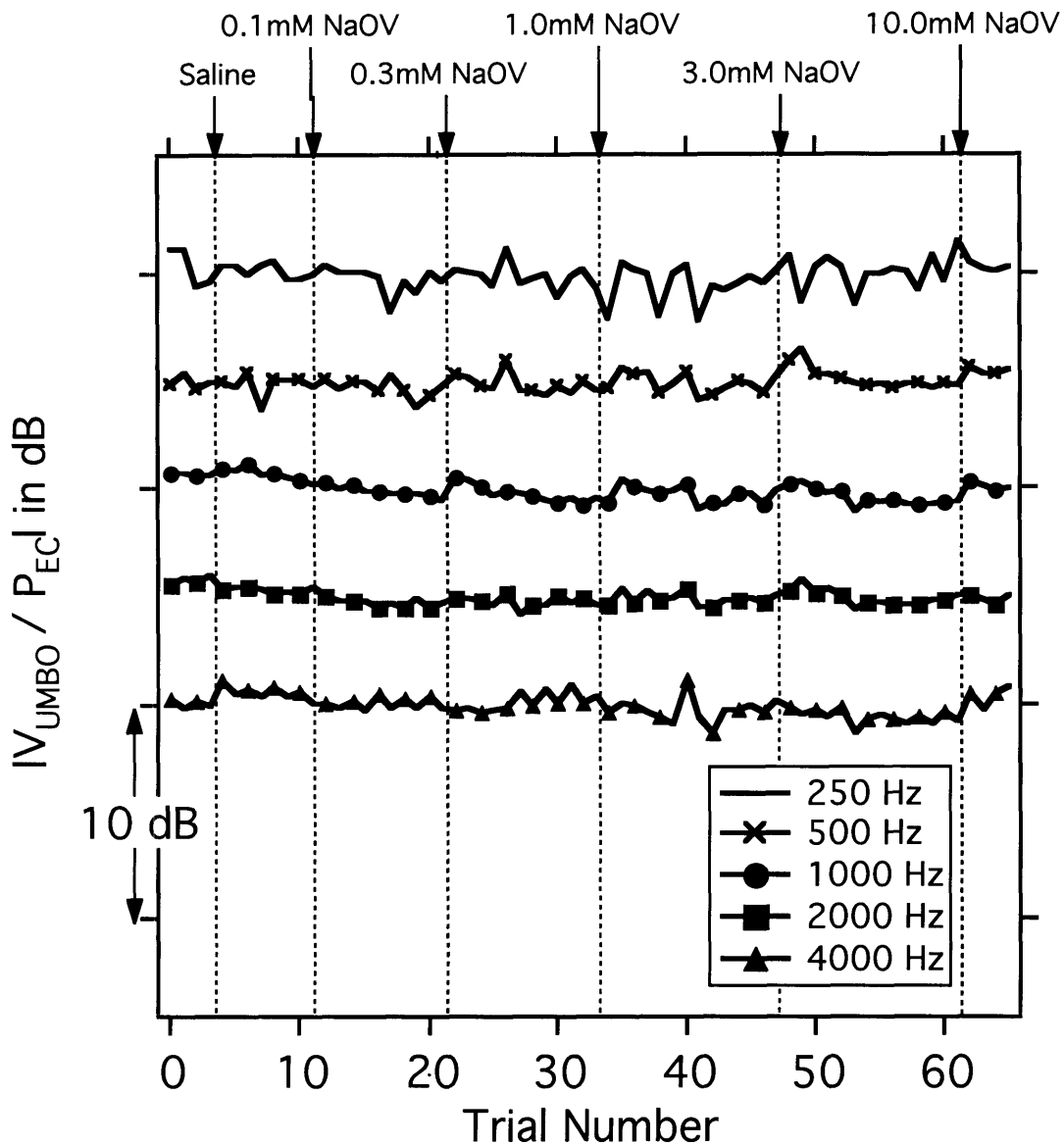


Figure 3.13. The time-dependent $|H|$ response for Gerbil 040713 at 250 Hz, 500 Hz, 1000 Hz, 2000 Hz, and 4000 Hz. Applications of saline, 0.1mM NaOV, 0.3mM NaOV, 1.0mM NaOV and 10.0mM NaOV were not associated with a change in $|H|$. Application of 3.0mM NaOV was associated with an increase in $|H|$ at 500 Hz.

Figure 3.14

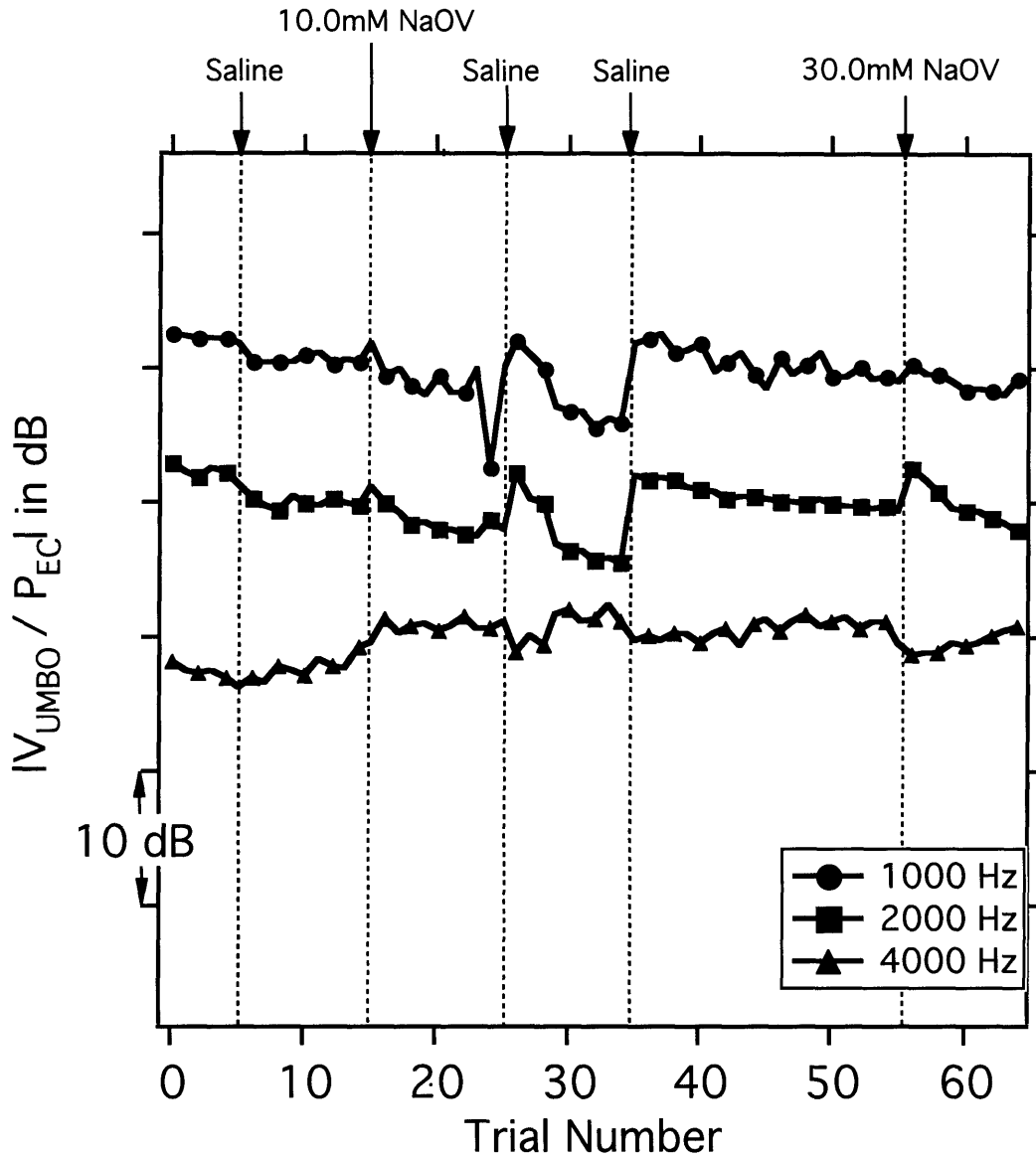


Figure 3.14. The time-dependent $|H|$ response for Gerbil 040810 at 1000 Hz, 2000 Hz, and 4000 Hz. A transient increase in $|H|$ associated with the second saline application was observed at 1000 Hz and 2000 Hz. The third saline application was associated with a sustained increase in $|H|$ at 1000 Hz and 2000 Hz. Application of 30.0mM NaOV was associated with an increase in $|H|$ at 2000 Hz and a decrease in $|H|$ at 4000 Hz.

Figure 3.15

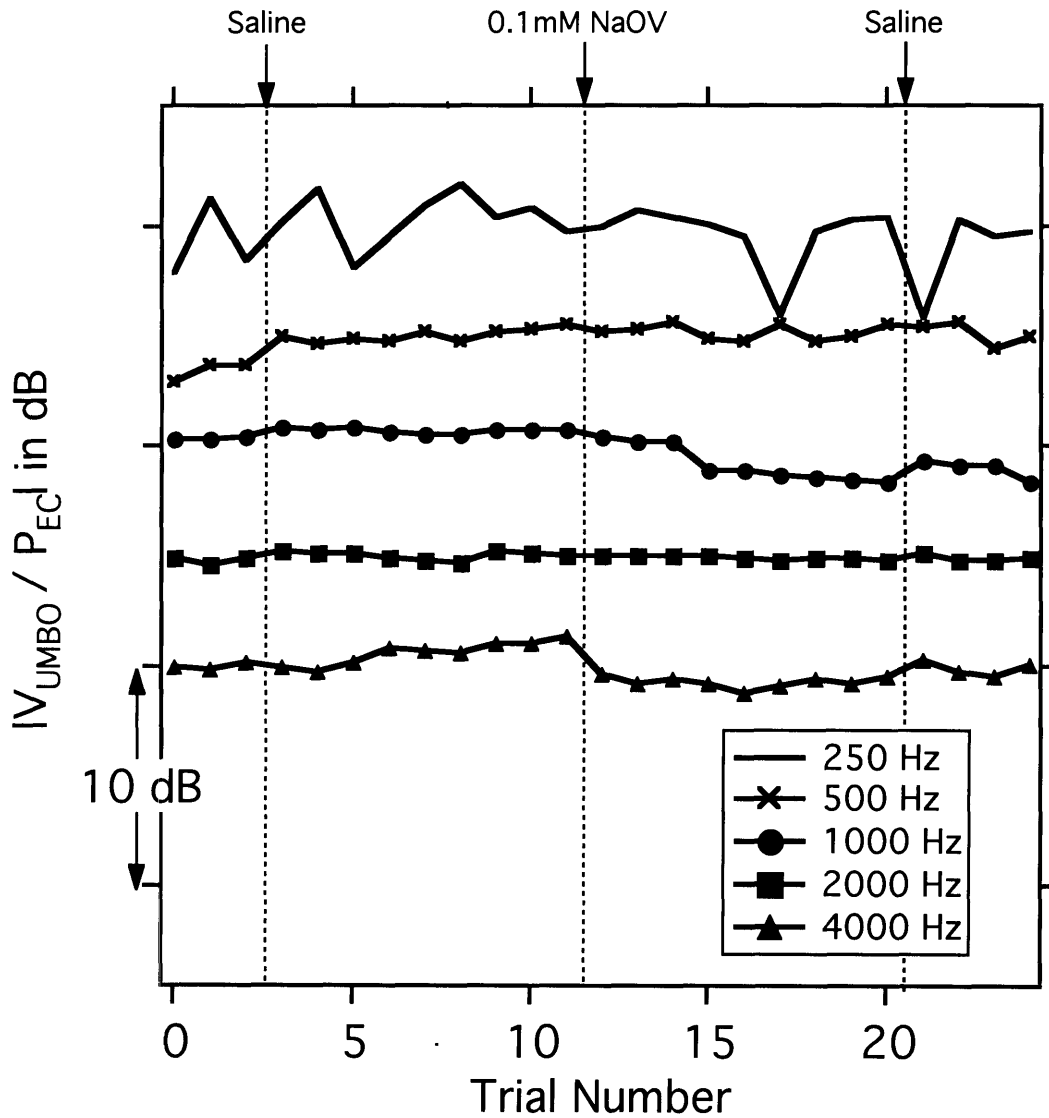


Figure 3.15. The time-dependent $|H|$ response for Gerbil 040726 at 250 Hz, 500 Hz, 1000 Hz, 2000 Hz, and 4000 Hz. Application of 0.1mM NaOV was associated with a decrease in $|H|$ at 4000 Hz.

Figure 3.16

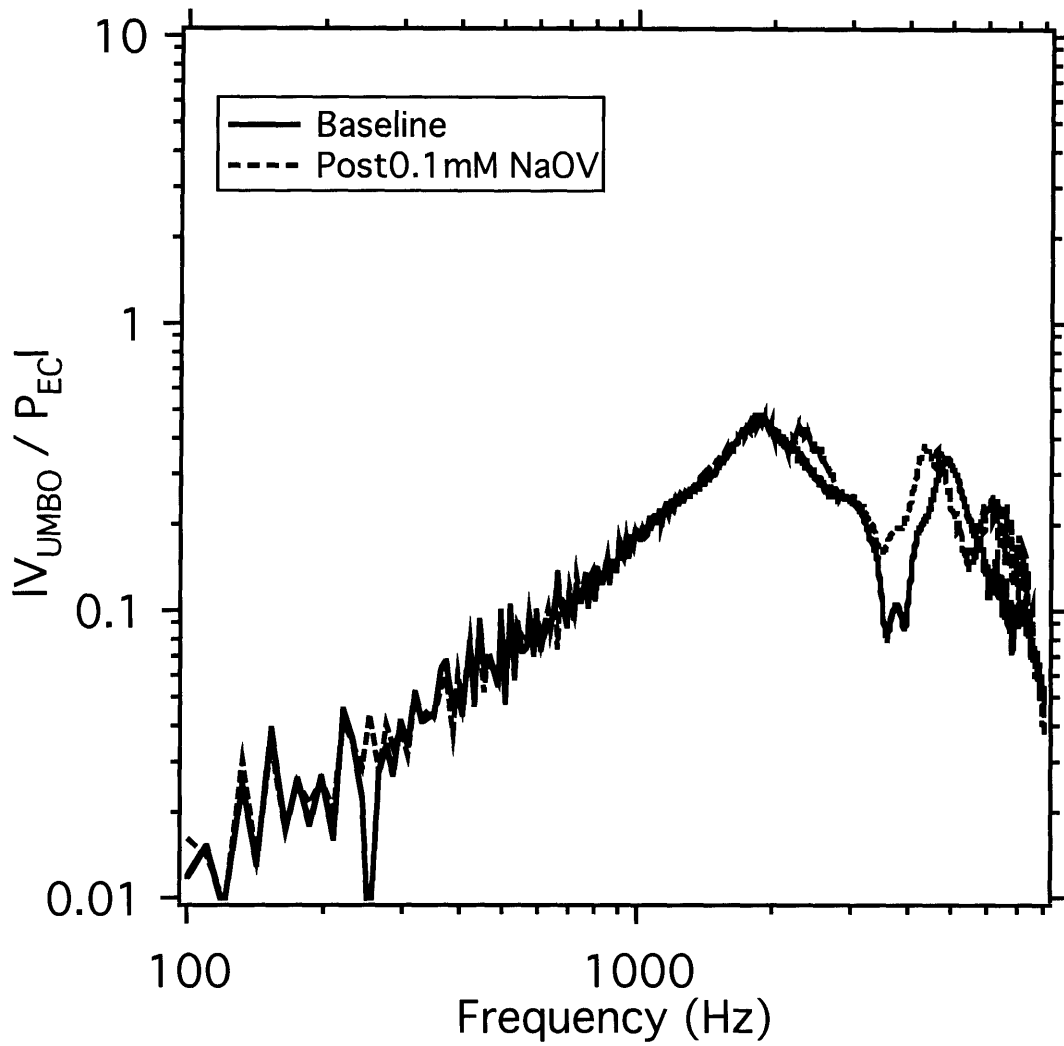


Figure 3.16. The frequency-dependent $|H|$ response for Gerbil 040702_left. The solid black line is the baseline response and the dotted black line is the 15-minute post-0.1mM NaOV response. There is a large increase in $|H|$ between 3000 Hz and 5000 Hz.

Figure 3.17

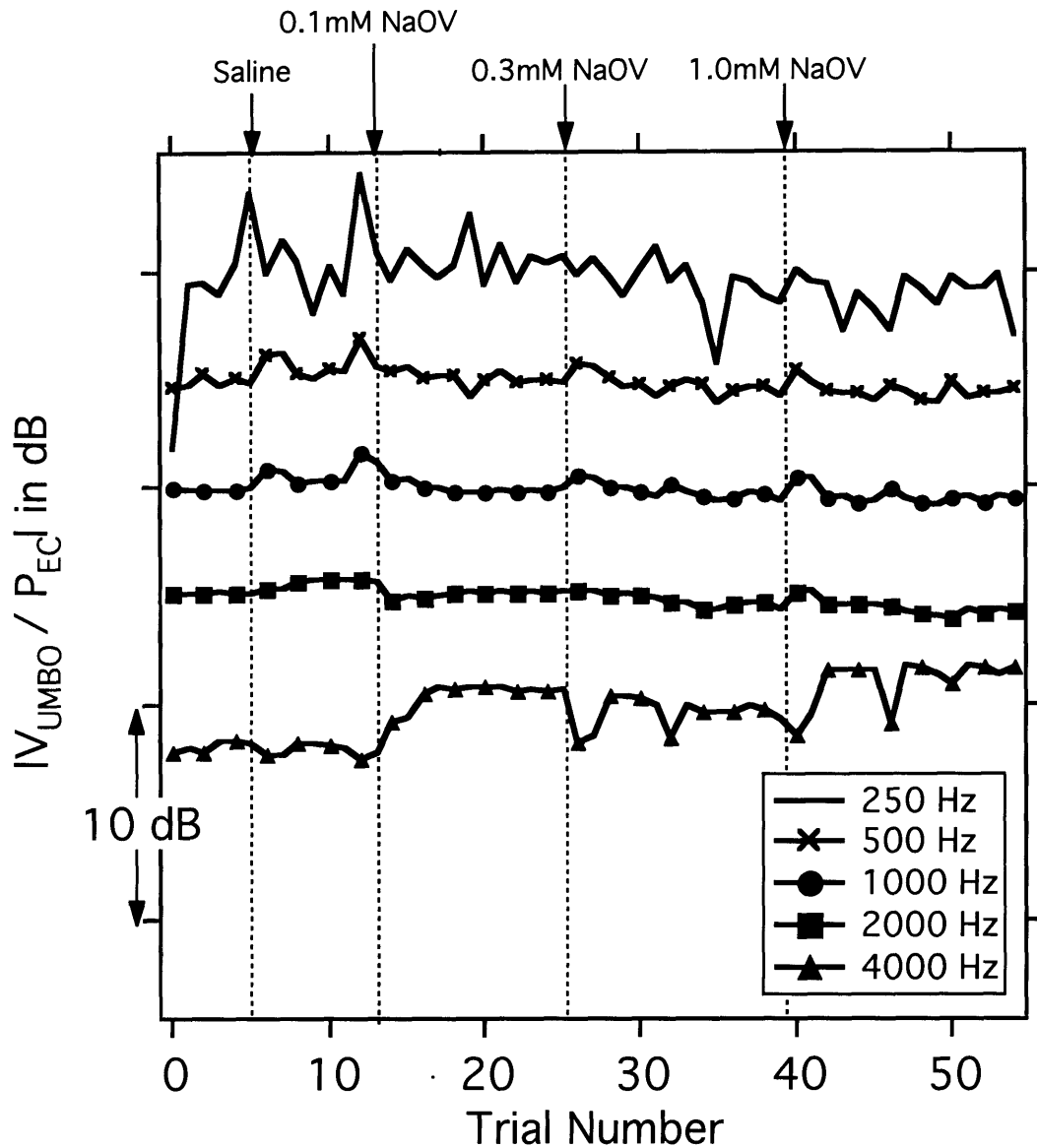


Figure 3.17. The time-dependent $[H]$ response for Gerbil 040702_left at 250 Hz, 500 Hz, 1000 Hz, 2000 Hz, and 4000 Hz. Applications of 0.1mM NaOV, 0.3mM NaOV, and 1.0mM NaOV are associated with sustained and transient increases and decrease in $[H]$ at 4000 Hz. The response pattern exhibits dose-dependent characteristics at 4000 Hz.

Figure 3.18

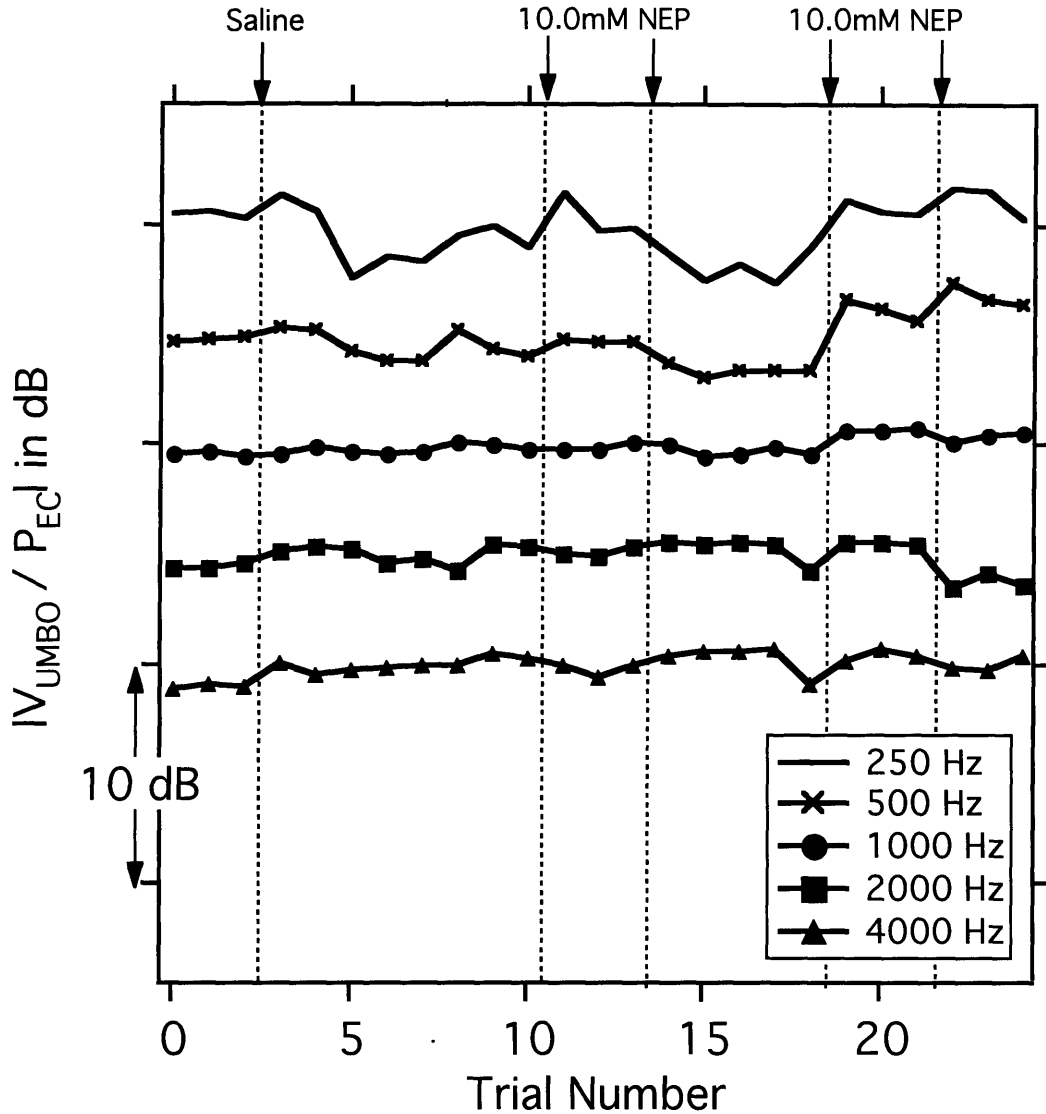


Figure 3.18. The time-dependent $|H|$ response for Gerbil 040819 at 250 Hz, 500 Hz, 1000 Hz, and 4000 Hz. The first application of 10.0mM NEP was associated with an increase in $|H|$ at 250 Hz. The third application of 10.0mM NEP was associated with an increase in $|H|$ at all five frequencies. The fourth application of 10.0mM NEP was associated with an increase in $|H|$ at 250 Hz and 500 Hz and a decrease in $|H|$ at 1000 Hz and 2000 Hz.

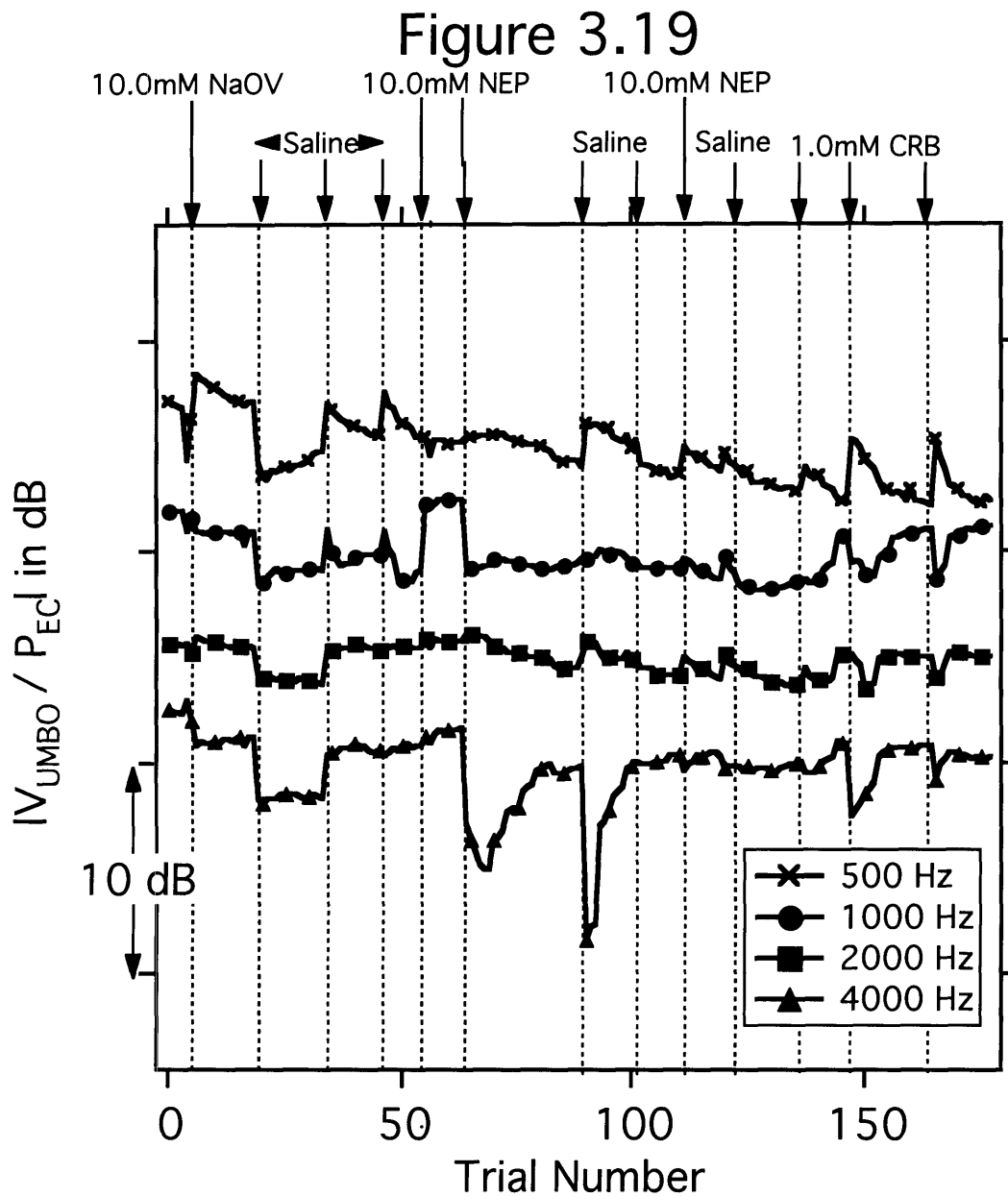


Figure 3.19. The time-dependent $[H]$ response for Gerbil 040818 at 500 Hz, 1000 Hz, 2000 Hz, and 4000 Hz. Applications of saline, 10.0mM NaOV, 10.0mM NEP, and 1.0mM CRB were associated with transient and sustained increases and decreases in $[H]$ across all four frequencies examined.