A transneuronal analysis of the olivocochlear and the middle ear muscle reflex pathways

Sudeep Mukerji

Dissertation for the degree of philosophiae doctor (PhD)
at the University of Bergen

2016

Dissertation date: 29.04.16

“There is nothing noble in being superior to your fellow man; true nobility is being superior to your former self.”

-Ernest Hemmingway

CONTENTS

1. ACKNOWLEDGEMENTS

It has been an absolute pleasure to spend four formative years at the Massachusetts Eye & Ear Infirmary/Harvard Medical School conducting basic science research to further understand the intricacies of the auditory brainstem. After completing my medical studies at the Royal College of Surgeons-Ireland in 2005, I fulfilled a medical and surgical internship in Dublin, Ireland until 2006. After which, I was appointed as a post doctorate research fellow by Dr. Daniel Lee (Associate Professor in Otology, Harvard Medical School) and Dr. M Christian Brown (Associate Professor in Otology, Harvard Medical School/Massachusetts Institute of Technology). During our time together, we immersed ourselves in the study off the neurophysiology of the mammalian auditory reflex pathway. Both Dr. Lee and Dr. Brown have been instrumental as mentors and trusted friends.

After completing my fellowship, I worked as a general surgical resident for a year at the Brigham & Woman's Hospital, Boston (USA) before returning to Norway to pursue my goal to be a trained otolaryngology specialist in Norway. My first appointment upon returning was a position at the otolaryngology department in Stavanger University Hospital. The chairman of the department, Dr. Jørg Tørpel, showed full appreciation for our previous work and encouraged me wholeheartedly to pursue my goals in converting our research results into a national recognized PhD. After over 3 years in Stavanger, I was offered a position at the otolaryngology/ Head & Neck surgical department at the University of North Norway-Tromso to complete the final stages of my surgical training. It was here that my new chairman, Dr. Torgrim Fuhr, graciously allowed me to continue working on my PhD project parallel to the requirements of my clinical duties. Further more and perhaps most importantly, Professor Nils Erik Gilhus (Professor of Neurology at the University of Bergen, Norway) has been extremely important in providing the structure, guidance and expertise in this endeavor, demonstrating immense patience, time and graciousness towards my efforts.

The decision to initially study the middle ear muscle reflex pathway, and more specifically the tensor tympani component of the reflex, was made by Dr. Daniel Lee, who had a particular research interest in this topic. Dr. Lee knew beforehand that I had little experience in basic science research and he helped to develop within me, the necessary traits required to be an effective clinician, academician and scientist. Through Dr. Lee, I have also been introduced to many clinicians and experts in the field of brainstem research, who educated me on the importance of combining academic science with a clinical application. Under the support and supervision of Dr. M. Christian Brown, I gained a better appreciation for the scientific method and the relentless pursuit for excellence in all areas of basic science research. Dr. Brown's patience and mantra of "never be satisfied" are ideals that I carry with myself to this day. Dr. Jørg Tørpel has been a constant source of encouragement and I am grateful for the advice that he has provided during this process. A special and profound consideration must go to my

primary mentor and advisor, Prof. Gilhus who has been the decisive factor that made the goal of achieving my goal an attainable possibility.

Through our collaborative work in constructing the research into a workable thesis, Prof. Gilhus has been vital in allotting me the mental tools and constructive critique that has allowed me to come this far. To present my work for acceptance within a prestigious academic environment in the country of my birth, Norway, has been a secret wish of mine returning to Norway and I must therefore also thank Dr. Lee, Dr. Brown, Dr. Tørpel and Dr. Torgrim Fuhr for making this possible. Gratitude must also be spread evenly towards the people who made the PhD practically possible: Leah Wanjiku Junge, Alanna Windsor, Jessica Levine and Marie Drottar. A special gratitude must be given to my parents, Kalyan and Nita Mukerji, and my wife Marte Thomsen Mukerji. Without their support, patience, encouragement and sacrifice, none of this would have been possible and for this reason alone, I dedicate the following dissertation to them. Thank you.

2. SCIENTIFIC ENVIRONMENT

The studies included in this work have been carried out under the supervision of Dr. Daniel Lee MD and Dr. M. Christian Brown at the Massachusetts Eye and Ear Infirmary (Harvard Medical University). Both studies were supported by various NIDCD (National Institute on Deafness and other Communication Disorders) grants. The construction and application of the results and conclusions in this thesis have been accomplished under the guidance and supervision of Professor Nils Erik Gilhus MD who is associated with the Department of Neurology at the University of Bergen. The laboratory work was done at the Eaton Peabody Laboratory situated on the fourth floor of the Massachusetts Eye and Ear Infirmary under the Department of Otolaryngology and Head & Neck Surgery / Harvard Medical University. The laboratory is run by affiliates to both Harvard Medical University and the Massachusetts Institute for Technology and is headed by Professor Charles Liberman. In this laboratory, clinical doctors work alongside a wide range of academic scientists within the fields of medicine, surgery, molecular biology, electrical engineering, acoustic scientists and basic science research.

The placement of the laboratory within a hospital, in a rich academic environment and in close alliance with a world renowned technological institute, makes it well suited for carrying out clinical based research using new and advanced methodologies such as those presented in this thesis. It was also possible to conduct parallel research into the historical implications of my study through the access of original historical documents at one of the centers for rare and classical books located in the Harvard University campus.

I was granted a four years post doctorate research fellowship under the immediate tutelage of associate professors Dr. Daniel Lee MD and Dr. M. Christian Brown through self- finance and later through various grants from the United States Government and the NIDCD. After the acceptance of four research papers for publication in diverse international scientific journals, I was afforded the opportunity in 2014 to participate in the PhD program conducted by the University of Bergen under the mentorship and guidance of Professor Nils Erik Gilhus. Since 2011, I have been working in various institutes in Norway as a doctor in specialization within the field of Otolaryngology-Head & Neck Surgery (Stavanger University Hospital; 2011-2014, University Hospital of North Norway in Tromsø; 2014-current). Writing my thesis has been undertaken in parallel to my clinical training as a full time specialist candidate within the field of Otolaryngology and Head & Neck Surgery that I aim to complete in 2016.

3. ABSTRACT

Background

In the auditory brainstem of mammals, there are two main descending reflex systems to the auditory system; The middle ear muscle reflex and the olivocochlear reflex. The two middle ear muscles participating in the middle ear muscle reflex are the stapedius and the tensor tympani. In man, the stapedius is known to react to strong low frequency acoustic stimulation, enacting forces perpendicular to the stapes superstructure, increasing middle ear impedance and reducing the intensity of acoustic energy arriving at the cochlea of the inner ear. Unlike the stapedius, the tensor tympani muscle has been proven to contract in response to self-generated noise such as chewing, swallowing and other non-auditory stimuli. The first theories on tensor tympani function were created by the a 16th Century Italian anatomist and scientist called Hieronymous Fabricius (1533-1619). He was the first to allude to both an auditory and non-auditory role of the tensor tympani muscle in humans. Since his work, many theories have been created founded on an evolving ability to analyze the components of the middle ear reflex pathways of the brainstem using various labeling techniques. It is now known that transduction of sound happens in the cochlea, causing an action potential that is sent along the auditory nerve to the cochlear nucleus in the brainstem. The cochlear nucleus is the first relay station for all ascending sound information originating in the ear. Unknown interneurons in the ventral cochlear nucleus then spread either directly or indirectly to the multiple middle ear muscle motoneurons located elsewhere in the brainstem. These motoneurons provide efferent innervation to the stapedius and the tensor tympani. There are many interesting differences among species in the acoustic thresholds for contraction of the middle ear muscles, which may be a reflection of underlying anatomical and physiological differences such as the number of tensor tympani muscle motoneurons. The goal of one of our research studies was to investigate the quantity, location and morphological characteristics of the tensor tympani motoneurons in the mouse model. Although the ascending and descending limbs of these reflex pathways have been described, the identity of the reflex interneurons within the reflex pathway is still unknown, as are the sources of modulatory inputs to these pathways. Olivocochlear neurons participate in the olivocochlear reflex pathway. They react to acoustic stimulation and provide descending input that controls auditory processing in the cochlea. As in the middle ear muscle reflex, the identities of these neurons in the pathways providing inputs to olivocochlear neurons are also incompletely understood and similar labeling techniques were used to further study these interneurons. Furthermore, we relate our findings to the unpublished results off recent experiments that used infrared light as a means of stimulating the auditory brainstem as a possible technology in future clinical applications of brainstem implants.

Materials and methods

This work consists primarily of four papers of which two are based on research that focuses on the anatomical geography (and postulated function) of the middle ear and the olivocochlear reflex pathways. The animal models in each investigation were mice

and guinea pig. For the tensor tympani muscle reflex experiments, we used the chemical trans-synaptic tracer called Fluorogold to retrogradely label cell bodies and their dendrites in mice. For the olivocochlear reflex experiments, we also used a retrograde transneuronal tracer but in the form of a pseudorabies virus (Bartha strain, expressing green fluorescent protein) to label neurons and their input in guinea pigs. These animal models have become the most common subjects for auditory and neuroscience research based on many factors, both biological and practical. The mouse and guinea pig were chosen because of the large availability of genetically altered strains in neuroscience research. Their relatively short lifespan renders them preferable for studies on the effects of aging. Furthermore, the very high frequency range of the hearing in mice vs. the low-frequency effects of middle ear muscle contraction makes it interesting to speculate on the functional roles of middle ear muscles in this species. The aim of the scientific papers was to provide an overview of the middle ear muscle reflex anatomy and physiology, to present new data on the middle ear muscle reflex anatomy and physiology, to describe the clinical implications of our research and to dedicate some attention to the historical efforts of research on the middle ear muscles, especially the tensor tympani. The latter was achieved by studying the original theories presented on tensor tympani function postulated by a renowned Italian anatomist/scientist named Hieronymous Fabricius (1533-1619). These theories, translated from Latin, were analyzed from his book *"De Visione, Voce et Auditu"* (The vision, voice and hearing) first published in 1600 and access to which was gained with scheduled permission from the Harvard Center for Rare Books, Cambridge (Massachusetts, USA).

Results and conclusions

After injections of Fluorogold into the tensor tympani muscle, a column of labeled tensor tympani motoneurons (TTMNs) was identified ventro-lateral to the ipsilateral trigeminal nucleus. The labeled TTMNs were classified according to their morphological characteristics into three subtypes: "octopus-like", "fusiform" and "stellate", suggesting underlying differences in function. All three subtypes formed sparsely branched and radiating dendrites, some longer than 600 μm. Dendrites were longest and most numerous in the dorso-medial direction, stretching into non-auditory regions of the brainstem. The long dendrites and the various subtypes of TTMNs support the idea that contraction of the tensor tympani muscle can be secondary to multiple non-auditory inputs. Our findings agree with past experiments showing that the labeled TTMNs were found just outside the trigeminal motor nucleus, probably forming part of a separate "tensor tympani motor nucleus of V". This separate nucleus was distinct from the trigeminal motor nucleus in term of cellular composition and orientation. To explore the olivocochlear pathways, the retrograde transneuronal tracer pseudorabies virus (Bartha strain, expressing green fluorescent protein) was used successfully to label neurons and their inputs in guinea pigs. Labeling of olivocochlear neurons started on the first day after injection into the cochlea. On the second day (and for longer survival times), transneuronal labeling spread to the cochlear nucleus, inferior colliculus, and other brainstem areas. There was a relationship between the numbers of these transneuronally labeled neurons and the number of labeled medial olivocochlear neurons, implying that the spread of labeling proceeds predominantly via synapses on the medial olivocochlear neurons. In the cochlear nucleus, the transneuronally labeled neurons were

classified as “multipolar” cells including the subtype known as “planar” cells. In the central nucleus of the inferior colliculus, transneuronally labeled neurons were of two principal types: neurons with disc-shaped dendritic fields and neurons with dendrites in a stellate pattern. Transneuronal labeling was also observed in pyramidal cells in the auditory cortex and in centers not typically associated with the auditory pathway such as the pontine reticular formation, subcoerulean nucleus, and the pontine dorsal raphe. These data presents us more information on the identity of neurons providing input to auditory neurons, which are located in auditory as well as non-auditory centers. Additionally, we learnt from translated written accounts that Fabricius was a pioneer in approaching anatomy from a structure-function relationship and that he was an active proponent for improving the learning environment for students. The writings of Fabricius on the middle ear also provided the foundation for modern ideas on the role of the tensor tympani in mammals. He was also the first to propose a non-auditory function to this middle ear muscle.

4. LIST OF PUBLICATIONS

Paper 1:

A morphologic study of Fluorogold-labeled tensor tympani motoneurons in mice

Sudeep Mukerji, Daniel J Lee & M. Christian Brown.

Brain Research 2009; 1278:59-65

Paper 2:

Identification of inputs to olivocochlear neurons using transneuronal labeling with pseudorabies virus (PRV)

M. Christian Brown, Sudeep Mukerji, Marie Drottar, Alanna Windsor & Daniel J Lee.

Journal of the Association for Research in Otolaryngology 2013; 14:703-717

Paper 3:

De Visione, Voce et Auditu: the contribution of Hieronymous Fabricius to our understanding of tensor tympani function

Sudeep Mukerji & Daniel J Lee.

Otolology & Neurotology 2010; 31:536-43

Paper 4:

Auditory brainstem circuits mediating the middle ear muscle reflex

Sudeep Mukerji & Daniel J Lee.

Trends in Amplification 2010; 14:170-191

5. ABBREVIATIONS

ABC	Avidin Biotin Complex
ABI	Auditory Brainstem Implant
AC	Auditory cortex
AN	Auditory Nerve
AVCN	Antero-ventral cochlear nucleus
BSA	Bovine serum albumin
C	Cochlea
CN	Cochlear Nucleus
CN7	Facial nerve (Cranial Nerve 7)
CT	Computed Tomography
DCN	Dorsal cochlear nucleus
DCV	Dense core vesicle
DMPO	Dorso-medial periolivary nucleus
DPOE	Distortion product otoacoustic emissions
GrC	Granular layer of cochlear nuclei
GFP	Green Florescent Protein
GP	Guinea pig
IC	Inferior colliculus
IM	Intra-muscular
INS	Infra-red neural stimulation
IP	Intra-peritoneal
LC	Locus subcoerulean
LOC	Lateral olivocochlear
LSO	Lateral superior olive
MEMs	Middle ear muscles
MOC	Medial olivocochlear
MO5	Motor nucleus of 5^{th} cranial nerve (Trigeminal)
MNTB	Medial nucleus of the trapezoid body
MSO	Medial superior olive
NBS	Normal bovine serum
NF-2	Neurofibromatosis Type 2
NGS	Normal goat serum
NIDCD	National Institute for Deafness and other Communicative Disorders
NIH	National Institute for Health
NLL	Nuclei of lateral lemniscus
OC	Olivocochlear
oABR	Optically evoked Auditory Brainstem Responses
PB	Phosphate buffer
PBS	Phosphate buffered saline
PO	Peri-olivary nucleus
PP	Pyramidal process

PRV	Pseudo-Rabies virus
PVCN	Postero-Ventral cochlear nucleus
Raphe	Dorsal Raphe of the brainstem
Retic	Reticular formation of the brainstem
Sgl	Superficial layer of granule cells.
SMNs	Stapedial motoneurons
SOC	Superior olivary complex
St	Stapedius
TT	Tensor Tympani
TTMNs	Tensor tympani motoneurons
VCN	Ventral cochlear nucleus
VNR	Vestibular Nerve Root
VNTB	Ventral nucleus of the trapezoid body

6. INTRODUCTION

6.1 MIDDLE EAR MUSCLES

6.1.1 Identity of the middle ear muscles

The tensor tympani and the stapedius are the two middle ear muscles that participate as the target organs in the middle ear muscle (MEM) reflex. The stapedius and tensor tympani muscles contract to regulate the transmission of sound energy traveling to the inner ear through the middle ear. Both muscles are striated muscles and are arranged as anatomical antagonists that contract synergistically to decrease sound transmission through the middle ear and ossicular chain **(Figure 1A).**

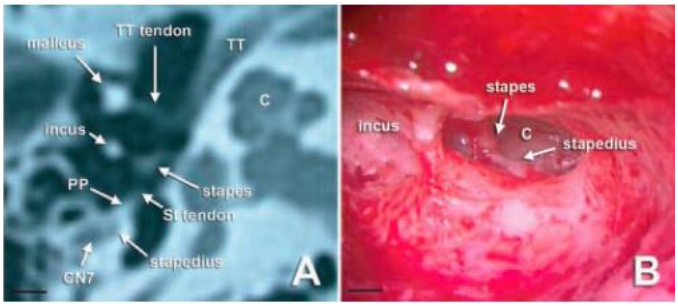

Figure 1. Middle ear muscles in man
A (left panel): High-resolution computed tomography (CT) of the right temporal bone: axial plane. In this CT image, both the stapedius (St) and tensor tympani (TT) are seen in the middle ear, attached to the stapes and malleus, respectively. The St tendon arises from the pyramidal process (PP) and the TT tendon arises from the cochleariform process (not seen). Scale bar: 2 mm. **B (right panel):** Intra-operative photograph, right ear, following completion of a facial recess (posterior tympanotomy) approach to expose the middle ear and cochlea prior to cochlear implantation. The stapedius is clearly seen with the tendon attached to the posterior neck of the stapes superstructure. Scale bar: 5 mm. C, cochlea; CN7, facial nerve.

6.1.2 Stapedius – anatomy, innervation and histology

The stapedius measures approximately 6 mm in length (Seikel, King, & Drumright, 2000), arises from the pyramidal process of the postero-superior mesotympanum with the tendon attaching to the posterior neck of the stapes capitulum (Moller, 2006; Seikel et al., 2000; **Figure 1B).** The stapedius is innervated by the stapedial branch of the facial nerve (cranial nerve VII; Politzer, 1861). Contraction of this muscle results in posterior rotation and stiffening of the stapes superstructure at the oval window. The stapedius consists of a large proportion of type II muscle fibers that are rich in myosin (Dammeijer, van Dijk, Manni, & van Mameren, 2006), are multinucleated, are

densely concentrated with ribosomes, and contain a lower concentration of potassium relative to sodium and calcium (Anniko & Wroblewski, 1981). These characteristics enable the stapedius to have a high oxidative capacity that allows it to contract quickly and repeatedly for long periods of time without fatigue (Lyon & Malmgren, 1982).

6.1.3 Tensor tympani – anatomy, innervation and histology

The tensor tympani is a slender, feather-shaped muscle measuring approximately 25 mm in length (Seikel et al., 2000) and is comprised of short striated muscle cells arranged in parallel fibers (Neergaard, Andersen, Hansen, & Jepsen, 1964), allowing this muscle to exert tension with minimal displacement **(Figure 1A)**. The tensor tympani originates from the cartilaginous portion of the Eustachian tube, courses through a bony canal in the wall of the anterior middle ear cavity, and attaches to the neck of the manubrium of the malleus (Moller, 2006). The tensor tympani is innervated by the "nerve to the tensor tympani" via the otic ganglion (Girardet, 1960), a branch of the mandibular division of the trigeminal nerve (cranial nerve V; Politzer, 1861; Shankland, 2001). Contraction of the tensor tympani muscle pulls the malleus in an anteromedial (inward) direction (Neergaard et al., 1964). A bony partition separates the tensor tympani from the Eustachian tube. The precise reason for this is unclear although it has been theorized that the partition insulates the muscle from vibrations that interfere with sound perception.

The tensor tympani muscle has fewer type II muscle fibers than the stapedius muscle (Lyon & Malmgren, 1988). More than one third of the tendon contains elastic tissue, dampening the ossicles during excessive stimulation (Lyon & Malmgren, 1988; Neergaard et al., 1964). A large amount of fat is also found in the human tensor tympani, but the physiological significance of this is uncertain (Neergaard et al., 1964). There is a dense concentration of motor and proprioceptive nerve fibers in this muscle. These fibers are thinner than other skeletal muscle nerves but still ensure rapid conduction velocities because of their shorter axons (Girardet, 1960). The postsynaptic regions are smaller when compared with stapedial fibers and with less mitochondrial activity in the motor end plates (van den Berge & Wirtz, 1989).

6.1.4 Middle ear muscles: a historical perspective

The Italian Renaissance (14th-16th century) was a period marked by significant advancements in the knowledge of temporal bone anatomy, including the discovery of the stapedius and tensor tympani muscles. The first published observation of the MEMs are credited to Italian anatomists Constantius Varolius (1543-1575; Politzer, 1981), Andreas Vesalius (1514-1564; Vesalius, 1725) and Bartolomeo Eustachius (1500-1574; Eustachii, 1564). Vesalius elevated anatomy to an empirical science (Geiringer, 1970; Mukerji & Lee, 2010) and pioneered anatomic teaching methods as human cadavers gradually replaced animal dissection. Specifically, he made the teaching of anatomy more interactive by using direct observation as the dominant teaching aid. An ardent proponent of Vesalius's systematic approach was the Italian anatomist Hieronymus Fabricius (1533-1619) (Cunningham, 1985). Fabricius **(Figure 2)** was one of the first scientists to introduce structure– function relationships in anatomical research. In addition to

publishing his observations of venous valves and human fetal formation, Fabricius also proposed the first theories on tensor tympani function. His approach represented a step forward in the study of gross anatomy to understand the "notia organorum tota" that is, the entire knowledge of the organ (Smith, Macchi, & Parenti, 2004) and his published observations attributed a protective and a non-auditory role to the tensor tympani muscle (Mukerji & Lee, 2010). Fabricius' theory of tensor tympani physiology was described in his treatise De Visione, Voce et Auditu (1600). He wrote that the tensor tympani protected the tympanic membrane from external (and internal) forces and helped in middle ear ventilation.

Figure 2: Hieronymous Fabricius (1533-1619). Courtesy of the Wellcome Library, London, UK.

In the 20th century, the development of the acoustic impedance measuring bridge by a Danish scientist named Otto Metz (1905-1993) allowed for the first objective measurements of middle ear function. Experiments on human subjects with various ear pathologies confirmed that the stapedius was the dominant sound evoked middle ear muscle. However, Fabricius' original theory on the proposed tensor tympani role in middle ear ventilation persists to this day based on recent physiologic, embryologic, and histological studies. Unlike the stapedius muscle, the function of the tensor tympani in humans has continued to be a subject of constant speculation since it's discovery by Vesalius. The tensor tympani will continue to be the topic of further historical and research enquiry and analysis within this thesis.

6.2 MIDDLE EAR MUSCLE FUNCTION

6.2.1 Role in acoustic overstimulation

In most mammals, both the tensor tympani and stapedius participate in the MEM reflex. The auditory (acoustic/ "sound induced") MEM reflex responds to sound by increasing middle ear pressure/impedance. This decreases acoustic overstimulation and reduces the masking effects of background noise on hearing. A change in acoustic impedance due to contraction of the MEMs results in a decrease in middle ear volume. Reduction in middle ear volume leads to an increase in the middle ear pressure, which can be detected as a change in acoustic impedance by inserting a probe into the external

auditory canal (Lilly, 1972). The resulting increase in impedance reduces the forward (and reverse) transmission of acoustic energy through the middle ear (Borg, 1971; Borg, Counter, & Rosler, 1984; Borg, Nilsson, & Engstrom, 1983; Moller, 1974).

6.2.2 Acoustic impedance

Impedance is a scientific engineering concept defined as the ratio of the acoustic pressure over the volume velocity generated by the acoustic pressure (Chien & Lee, 2009; Lilly, 1972). The first electro-acoustic device to measure the acoustic impedance in a clinical setting was developed in the middle of the 20th century by the German scientist Otto Metz (1905-1993) and was called the "Metz acoustic bridge" (Metz, 1946, 1952; Thomsen, 1999). Since then, acoustic impedance measurements have allowed for the indirect monitoring of MEM contraction for both research and diagnostic purposes (Metz, 1946).

6.2.3 Stapedius as the primary muscle in the MEM reflex

The first observation of a sound induced MEM reflex in mammals was made in dogs (Hensen, 1878) and then later in primates (Kato, 1913). Luascher (1930) then reported the first observations of sound produced stapedial contractions in humans through perforated eardrums. Data from hearing experiments using acoustic impedance have shown that the stapedius and not the tensor tympani is the primary MEM (Borg, 1972; Carmel & Starr, 1963; Liberman & Guinan, 1998; Neergaard et al., 1964). Unlike some animal models, where both the stapedius and tensor tympani contract to sound, the stapedius reflex was later accepted as the dominant sound-produced MEM reflex pathway in humans (Zakrisson & Borg,1974; Liberman & Guinan, 1998; Murata, Ito, Horikawa, & Minami, 1986).

6.2.4 Role of the stapedius muscle in the MEM reflex

Based on the research mentioned in *6.2.3*, two major functions of the stapedius reflex have been proposed; 1) Reduction of acoustic energy reaching the cochlea through the control of middle ear impedance (Borg, 1971; Moller, 1974) and 2) Prevention of the masking of speech frequencies through the high-pass filtering of low frequency sound (background noise). In both cases, the fundamental function of the stapedial reflex pathway appears to be protective. Contraction of the stapedius results in a frequency-dependent sound reduction in the presence of intense acoustic stimuli (Aiken et al., 2013). For example, human ears with an absent stapedius reflex (secondary to a facial nerve palsy) were reported to have suffered from more temporary hearing loss when exposed to noise compared with normal ears with an intact stapedius reflex (Zakrisson, Borg, Liden, & Nilsson, 1980). The MEM reflex also minimizes masking of speech frequencies by background noise, which is typically lower in frequency, thereby preserving speech discrimination in noisy environments (Borg & Zakrisson, 1974, 1975; Mahoney, Vernon, & Meikle, 1979; Moller, 1984; Pang & Peake, 1986; Stevens & Davis, 1938). Lastly, the stapedius may also contract to internally generated vocalization in humans to reduce excessive stimulation (Borg & Zakrisson, 1975).

6.2.5 Role of the tensor tympani muscle in the MEM reflex

The tensor tympani in humans is less acoustically responsive than the stapedius muscle. Electromyographic (EMG) recordings of tensor tympani muscles in humans have shown minimal electrical activity in response to sound presentation to both ears (Djupesland, 1964, 1967; Salomon, 1963; Jones et al., 2008). Patients suffering from a paralyzed stapedius muscle from facial palsy or stapes surgery but who had intact tensor tympani function were shown to have no detectable or absent MEM reflexes (Stach et al., 1984). This finding supports previous observations that the tensor tympani muscle plays a minimal role in the auditory MEM reflex pathways in humans.

6.2.6 Non-auditory related activities of the tensor tympani muscle

Tensor tympani muscle activity through changes in acoustic impedance has been observed during specific non-auditory behaviors in humans. Examples of such behaviors include tactile stimulation of the external auditory canal and face (Klockhoff, 1961), pneumatic pressure on eyelids (Klockhoff & Anderson, 1960), the sudden forced opening of closed eyelids (Terkildsen, 1960), swallowing (Wersall, 1958), and head movements (Carmel & Starr, 1963). Measurable activity of the tensor tympani muscle is also associated with the anticipation of loud sounds (Borg et al., 1984; Gelfand, 1984, 1998; Klockhoff & Anderson, 1960; Terkildsen, 1960) and the startle response (Borg et al., 1984; Gelfand, 1984; Moller, 1984). The tensor tympani is also thought to play an important role in middle ear ventilation based on the close histological and embryological resemblance to Eustachian tube structures, such as the tensor veli palatini (Doyle & Rood, 1980; Kierner, Mayer, & v. Kirschhofer, 2002; Rood & Doyle, 1978; Vacher, Guinan, & Kobler, 1989; Blanke et al., 2014).

6.3 MIDDLE EAR MUSCLE REFLEX PATHWAY

6.3.1 General structure of the MEM reflex pathway

The dominant auditory or sound induced MEM reflex pathway in mammals (including humans) is the stapedius reflex. The tensor tympani reflex plays a less acoustically driven role. The MEM reflex begins as sound presented to one or both ears **(Figures 3 & 4).** Intense, low frequency sound presented to the ear contracts the stapedius muscle in both ears **(Figure 3A)** and the tensor tympani in the stimulated ear. Therefore both reflexes begin with acoustic stimuli presented to the ear and the transduction of sound to the cochlea, a spiral shaped structure in the inner ear involved in auditory signal processing. This creates an action potential that is transmitted along the auditory nerve to the cochlear nucleus (CN) in the brainstem. The CN is located in the pontomedullary junction of the dorsolateral brainstem in humans (Adams, 1986; Haines & Lancon, 2003; Harrison & Feldman, 1970; Palmer, 1987). The CN is an important

structure in the MEM reflex because it is the first relay station for all ascending sound information originating in the ear. Although the ascending (sensory) and descending (motor) limbs of MEM reflex pathways have been well characterized, the identity of the reflex interneurons (intermediate neurons) that exist in the CN and the source of modulatory inputs to these pathways is still not known **(Figure 3: panel B & Figure 4).** These reflex interneurons in the CN then stimulate either directly or indirectly specialized efferent cells in the brainstem called motoneurons.

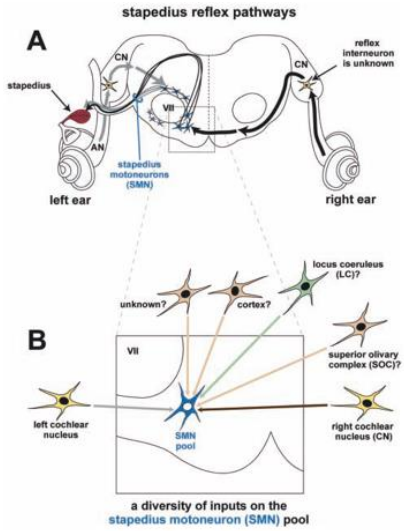

Figure 3. Stapedial reflex pathway
A: The uncrossed stapedius reflex (gray arrows) to the left ear is activated by left ear sound and stimulates the subpopulation of stapedius motoneurons (SMNs) that contract the stapedius muscle of the same ear (left ear). The cochlear nucleus (CN) reflex interneuron of the uncrossed pathway is shown by the large yellow star on the left. It is not clear whether it projects directly to the SMNs or whether there is an intervening synapse. The crossed MEM reflex to the left ear (black arrows) is activated by right ear sound and stimulates the subpopulation of SMNs that contract the stapedius muscle of the left ear. The CN reflex interneuron of the crossed pathway is shown by the large yellow star on the right. The crossed reflex pathway ending with contraction of the right stapedius is not shown. **B:** Magnified schematic showing the possible diversity of inputs on a representative SMN found ventromedial to the facial nucleus (VII). As shown in Panel A, SMNs respond to sound and likely receive input either directly or indirectly from both CN. Some patients can voluntarily contract their stapedius muscle and so these SMNs must also receive input from cortex. Transneuronal tracing using pseudo-rabies virus (Rouiller, Capt, Dolivo, & De Ribaupierre, 1989; Windsor, Roska, Brown, & Lee, 2007) has shown labeling in the superior olivary complex (SOC), locus coeruleus (LC), and inferior colliculus (IC) after injection into the stapedius muscle in rats. It is unknown whether these regions contain neurons that project directly or indirectly to SMNs.

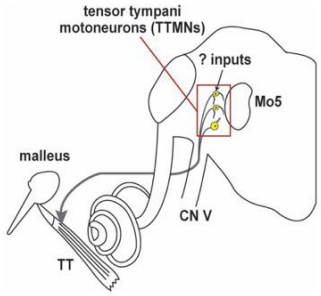

Figure 4. Tensor tympani (TT) reflex pathway
Note: The TT reflex to the left ear is activated by left ear sound and by neurons from elsewhere (?) and stimulates the population of tensor tympani motoneurons (TTMNs) that contract the TT muscle of the same ear (left ear). Based on recent pseudorabies viral tracing studies of the TT reflex by Windsor et al. (2007) and Billig, Yeager, Blikas, and Raz (2007), the interneurons that project from the cochlear nucleus to TTMNs may be multipolar cells located in the anterior ventral cochlear nuclei (AVCN). From the cochlear nuclei, neurons project either directly or indirectly to TTMN pool in rats. Last, efferent fibers (gray arrow) exit the brainstem to innervate the TT via the "nerve to the TT," a branch of the mandibular division of the trigeminal nerve. A range of inputs from the superior olivary complex, serotoninergic sources and higher brain centers are thought to descend on TTMNs. The variety of inputs may account for the multifunctional roles of this muscle in response to auditory and non-auditory stimuli (Borg, Counter, & Rosler, 1984; Gelfand, 1984, 1998; Moller, 1984; Nomura, Harada, & Fukaya, 1979; Stach, Jerger, & Jenkins, 1984). The crossed pathway by which the MEM reflex to the left ear is activated by right ear sound is not shown. Mo5, trigeminal motor nucleus; TT, tensor tympani; CN V, trigeminal nerve; TTMN, tensor tympani motoneurons.

6.3.2 Individual neural components of the MEM reflex pathway

The MEM reflex pathways have been shown to consist of three or four neurons **(Figures 3 & 4)** (Billig et al., 2007; Borg, 1973; Itoh et al., 1986; Itoh et al., 1987; D. J. Lee et al., 2006; Rouiller et al., 1986; Spangler, Henkel, & Miller, 1982) that comprise an ascending limb *(cochlea → auditory nerve → CN interneuron (→ superior olivary complex?)* and a descending limb *(motoneurons → stapedius or tensor tympani muscle*; Moller, 1984, 2006; Roeser, Valente, & Hosford-Dunn, 2000; Seikel et al., 2000). To date, the identification and organization of CN interneurons participating in the MEM reflex pathways have not been fully characterized. Viral transneuronal techniques have indicated a direct connection between the CN and the MEM reflex (Billig et al., 2007; Itoh et al., 1987) as well as the presence of an additional synapse after the CN (Rouiller et al., 1986, 1989; Windsor et al., 2007). Similar to the direct projections postulated for the medial olivocochlear complex (MOC) auditory efferent pathways (Thompson & Thompson, 1991; Ye, Machado, & Kim, 2000), the possibility of a direct link between the CN interneurons and MEM reflex is controversial (Itoh et al., 1986; Rouiller et al., 1986). The aim of this thesis is to characterize the MEM reflex motoneurons in greater detail on the basis of their location, morphological features, and dendritic characteristics.

6.4.1 MEM reflex motoneurons

Motoneurons supplying the stapedius and the tensor tympani muscles are called stapedial motoneurons (SMNs) and tensor tympani motoneurons (TTMNs) respectively. SMNs are located near the motor nuclei of the facial nerve and TTMNs are located in close proximity to the motor nuclei of the trigeminal nerve. The neural pathways from the CN interneurons to the motoneurons are still not yet fully understood. SMNs or TTMNs project to the middle ear along branches of the facial or trigeminal nerve, respectively, to innervate the stapedius muscle (stapedial nerve) or tensor tympani (nerve to tensor tympani). The afferent and efferent components of the MEM reflex have been well described in various studies (Borg, 1973; Guinan et al., 1989; Joseph et al., 1985; McCue & Guinan, 1988; Moller, 1984; Strutz, 1981; Strutz et al., 1988; Vacher et al., 1989, Venet et al., 2011).

6.4.2 Stapedius motoneurons (SMNs)

SMNs are located in close proximity to the facial motor nuclei bilaterally (**Figure 5;** Joseph et al., 1985; Rasmussen, 1946; Rouiller et al., 1989; Strominger et al., 1981; Windsor et al., 2007) and are distributed across the perifacial and periolivary regions in the cat (Lyon, 1978), and ventromedially and dorsomedially to the facial nuclei in guinea pigs (Strutz et al.,1988). SMNs have been reported to respond exclusively to either ipsilateral, contralateral, or bilateral acoustic stimulation (McCue & Guinan, 1988; Vacher et al., 1989) and are spatially organized around the facial motor nuclei according to their physiological responses to sound (Joseph et al., 1985; Lyon, 1978; Shaw & Baker, 1983). A similar spatial arrangement of neural innervation to the muscle fibers of the stapedius has not yet been described (Wiener-Vacher, Guinan, Kobler, & Norris, 1999). In cat, the stapedius has a higher innervation ratio than the tensor tympani (Blevins, 1964; Joseph et al., 1985). For instance, close to 1,100 SMNs in cat are found in the perifacial and peri-olivary regions of the brainstem in the cat (Joseph et al., 1985), and these SMNs supply approximately 1,730 stapedius muscle fibers. The stapedius muscle therefore has an innervation ratio of 1:1.6 (Blevins, 1964; Wiener-Vacher et al., 1999).

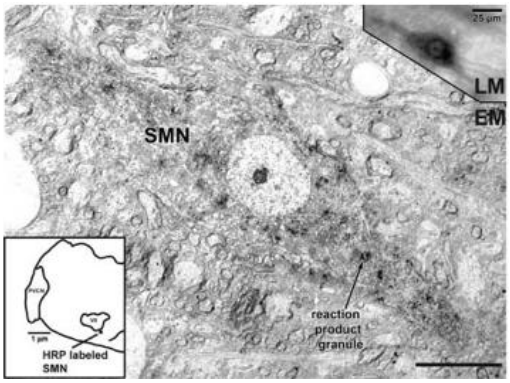

Figure 5. Horseradish peroxidase (HRP) labeled stapedius motoneuron (SMN) in a rat model
Note: Center image: low magnification electron micrograph of HRP-labeled SMN with black reaction product granules (scale bar = 10 mm). Lower left inset: drawing of the left half of a coronal section of rat brainstem demonstrating the location of the labeled SMN ventro-medial to the facial nerve motor nucleus (VII). PVCN-posteroventral cochlear nucleus. Upper right inset image: bright-field photomicrograph of the same epoxy-embedded labeled SMN. Source: D. J. Lee et al. (2008). Reprinted with permission from the Association for Research in Otolaryngology

6.4.3 Relationship of CN interneurons to SMNs

SMNs that project to the stapedius muscle receive input directly and indirectly from the CN, the first relay station for all ascending auditory information originating in the ear. A diversity of inputs have been theorized to descend on SMNs **(Figure 3B)** (D. J. Lee, Benson, & Brown, 2008), and some of these inputs may be non-auditory as a few patients are able to voluntarily contract their own stapedius muscle, suggesting projections to SMNs from the higher cerebral cortex. The stapedius is also known to contract in response to self-generated vocalization (Borg & Zakrisson, 1975) to minimize self-stimulation. Serotoninergic terminals on SMNs may modulate activity of the stapedius reflex (Thompson, Thompson, & Britton, 1998). Finally, higher brain centers may provide cortical control over contraction of the stapedius muscle (Borg et al., 1984; Gelfand, 1984; Gelfand, 1998; Moller, 1984; Nomura, Harada, & Fukaya, 1979; Stach et al., 1984). The sources of these descending inputs to SMNs are not known.
Following the transduction of acoustic stimuli by the inner hair cells, the action potential is propagated to the first-order neurons (spiral ganglion cells) and the auditory nerve to as yet unidentified interneurons in the ventral cochlear nucleus (VCN)(Fekete, 1984; D. J. Lee, de Venecia, Guinan, & Brown, 2006). CN interneurons found in the VCN (Billig, Yeager, Blikas, & Raz, 2007; Borg, 1973; D. J. Lee et al., 2006; Windsor, Roska, Brown, & Lee, 2007) project directly (or indirectly) onto SMNs that control the stapedius muscle.

6.4.4 Tensor tympani motoneurons (TTMNs)

Since the stapedius muscle plays a more dominant role than the tensor tympani in acoustically evoked middle ear muscle contraction, the SMNs have been studied more extensively (Borg, 1972; Brask, 1979; Ochi et al., 2002). However, there is a proven

large species-dependent range in acoustic threshold for middle ear muscle contraction for both stapedius and tensor tympani. This range has been studied with the aim of better understanding the role of the middle ear muscles particularly the tensor tympani muscle in humans. For example, rabbits and rats have very low acoustic thresholds (Borg and Moller, 1968; Relkin et al., 2005) and guinea pigs have very high thresholds (Avan and Loth, 1992). The conclusion from these comparative studies is that differences in middle ear muscle properties between species is a reflection of underlying differences in the anatomical (macroscopic & microscopic), neuro-anatomical and physiological characteristics of the tensor tympani. The light microscopic features of TTMNs have been studied in cats, guinea pigs, rats and mice (Lyon, 1975; Spangler et al., 1982; Mizuno et al., 1982; Friauf and Baker, 1985; Rouiller et al., 1986, Mukerji et al., 2008). TMNs are located as a narrow curvi-linear column in an area ventro-lateral to the ipsilateral trigeminal motor nucleus **(Figure 6).** The column extends along the entire rostro-caudal axis of the trigeminal motor nucleus.

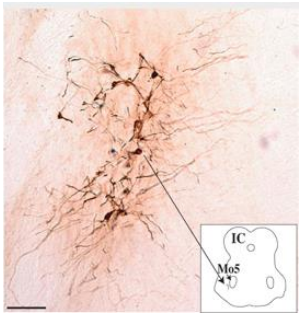

Figure 6. Micrograph of representative FG-labeled TTMNs
Labeled TTMNs in mice were organized as a curvi-linear column ventro-lateral to the trigeminal motor nucleus: Mo5 (see inset). IC: Inferior Colliculus. Scale bar: 100 μm.

6.4.5 Cell group K

Previous studies have referred to this pool of labeled TTMNs as being distinct from the trigeminal motor nucleus in terms of cell body size, location and function (Friauf and Baker, 1985). The area of labeled TTMNs has therefore been previously termed as being a "tensor tympani motor nucleus of V" rather than just an extension of the trigeminal motor nucleus (Hutson et al., 1979, Friauf and Baker, 1985). The location of TTMNs is thought to correlate to "Cell Group K," a specific area in the brainstem containing motoneurons that supply the masseter, digastric, and Eustachian tube muscles (Donga, Dubuc, Kolta, & Lund, 1992; Reuss, Kuhn, Windoffer, & Riemann, 2009; Saad, Dubuc, Westberg & Lund, 1999). Interestingly, it has been demonstrated in cat that only 700 TTMNs supply approximately 4,000 tensor tympani muscle fibers (Blevins, 1964; Shaw & Baker, 1983) to give the tensor tympani an innervation ratio of 1:5.7. Unlike SMNs, TTMNs have not been shown to be spatially located around the trigeminal motor nuclei according to their individual physiological responses to stimuli.

6.4.6 Different TTMN subtypes

Similar to SMNs, TTMNs have been observed to exhibit different morphologies based on the number and orientation of the primary dendrites that project from the cell body. In the research in mice that form the basis for this thesis, the following criteria and names were used; *"Stellate"* (star shaped) TTMNs had three or more dendrites leaving the cell body. *"Octopus-like"* TTMNs had two dendrites projecting from one side of the cell body. *"Fusiform"* TTMNs had two dendrites projecting from opposing poles of the cell body. This classification scheme has been used in studies of other species. "Fusiform", "elongated" and "lens-shaped" TTMNs were more common in cats and "ovoidal" and "pyramidal" TTMNs were the least common in cats (Friauf and Baker, 1985). "Stellate" TTMNs were more commonly seen in rats (Rouiller et al., 1986; Billig et al., 2007). These different subtypes may reflect differences in TTMN function similar to that seen in the CN, an important brainstem structure essential for central auditory processing of auditory impulses (Kiang et al., 1973; Rhode et al., 1983; Rouiller and Ryugo, 1984; Adams, 1986; Apostolides & Trussell, 2014). For example, different shapes and morphologies might account for the multiple functions of TTMNs in response to auditory and non-auditory stimuli. Alternatively, the different subtypes might innervate different types of muscle fibers, such as the slow, fast and medium twitch fibers. On histochemical analysis, the tensor tympani of rats is known to consist mainly of fast oxidative glycolytic fibers (Van den Berge and Wirtz, 1989).

6.4.7 Importance of TTMN dendrites

The TTMN dendrites are long, radiating and minimally branched **(Figure 7).** The study of dendrites is important because the orientation and distribution of dendritic spread have been to shown to influence neuronal response properties (Sotnikov, 2005; Berkowitz et al., 2006; Hickmott and Ethell, 2006; Saxon and Hopkins, 2006; Torres-Fernandez et al., 2007; Bergquist and Ludwig, 2008). Furthermore, proximal dendrites of TTMNs receive abundant synaptic input, but there is sparse innervation of the actual TTMN cell body (Lee et al., 2009). Distal dendrite synapses have not been investigated, but since the distal dendrites project extensively (Friauf and Baker, 1985), they present a large surface area on which to receive inputs. These inputs come from the cochlear nucleus (Billig et al., 2007), from serotoninergic sources (Thompson et al., 1998), and presumably from motor control areas. This may be reflected in the diversity of TT activity in response to both auditory and non-auditory stimuli. It is theorized that the distinct organization of TTMN dendrites reflects underlying physiological differences between the pool of TTMNs and the motoneurons of the trigeminal motor nucleus (Friauf and Baker, 1985).

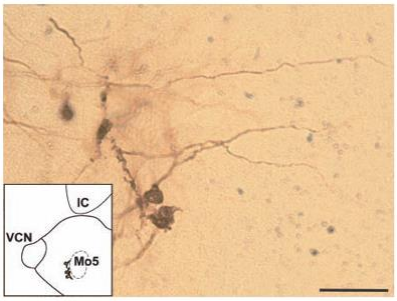

Figure 7. Fluorogold labeled TTMNs with dendrites
FG-labeled TTMNs with dendrites extending medially beyond the pool of labeled TTMNs. These TTMNs were located on the left side of the brainstem so the medial direction is toward the right. This section is located along the rostral–caudal distribution of labeled TTMNs at the position about 1/3 from the caudal end. Micrograph is a montage of two images taken of a single section at different focal planes. Scale bar: 50 μm.

6.5 SYNAPSES

6.5.1 Classification of MEM reflex motoneuron synaptic terminals

Until recently, the classification of synaptic terminal types was based on the subjective assessment of vesicle shape and size, which could be affected by factors such as packing density and comparison to vesicles in surrounding terminals (D. J. Lee et al., 2009, Brown et al., 2013). The quantification of morphometric differences between synaptic terminal types assists in a more precise assignment of each terminal type to a possible input source. Recent ultrastructural studies of synaptic terminals on MEM reflex motoneurons have examined studied the differences between terminal types based on synaptic vesicle morphometry (D. J. Lee et al., 2009; Brown et al., 2013). Morphometric studies of MEM reflex motoneuron terminals that include the mean vesicle area (nm^2) support the earlier subjective classification of the three common terminal types: small round, large round, and pleomorphic (D. J. Lee et al., 2009). Based on similar quantitative measurements, SMNs and TTMNs were shown to receive terminals of similar morphology. These motoneurons receive three common types of terminals as well as two rare types of terminals; those with heterogeneously sized vesicles and another terminal type packed with large dense core vesicles (DCVs; Benson et al., 2008; D. J. Lee et al., 2008, 2009). Because SMNs and TTMNs are found in separate regions of the brainstem and are unlikely to receive identical inputs, these observations suggest that similar neurotransmitters are used in these parallel MEM reflex circuits. Differences were seen between SMN and TTMN terminal types. For example, most large round SMNS terminals did not contain DCVs, whereas many large round TTMNs terminals did so (Benson et al., 2008; D. J. Lee et al., 2008).

Such differences probably reflect underlying functional differences, because the DCVs found in TTMN terminals (~80 nm diameter) can be associated with synapse assembly and plasticity (Sorra, Mishra, Kirov, & Harris, 2006). The surface areas of large round vesicles in SMN terminals (D. J. Lee et al., 2009) corresponded to a diameter comparable with similar vesicles found in globular bushy cell terminals in the medial nucleus of the trapezoid body (MNTB) in the superior olivary complex (Jean-Baptiste & Morest, 1975). Projection studies showed that labeled globular bushy cell axons projected to the caudal parts of the SOC and perifacial areas, a region containing SMNs (Spirou, Brownell, & Zidanic, 1990). There, they terminate in large endings (Smith & Brezinova, 1991) similar to the biggest large round terminals observed on SMNs (Benson et al., 2008; D. J. Lee et al., 2008).

6.5.2 Neurochemistry of motoneuron synaptic terminals

Auditory brainstem (including motoneuron) neurochemistry has been studied in great detail (Reuss, Al-Butmeh, & Riemann, 2008; Reuss et al., 2009, Kulesza et al., 2014). SMNs use Calcitonin gene–related peptide (CGRP) as a co transmitter to acetylcholine (Ach; Reuss et al., 2008). In contrast, TTMNs do not express CGRP but produce nitric oxide (Reuss et al., 2009). TTMNs also use acetylcholine, bombesin, cholecystokinin, and endorphin. Additionally, both SMNs and TTMNs were found to be closely related to structures chemically associated with neuroactive substances substance P and serotonin (Reuss et al., 2008, 2009; Thompson et al., 1998). Serotonin is believed to modulate MEM contractions during certain non-auditory activities such as chewing (Ramirez, Ballesteros, & Sandoval, 2007; Thompson et al., 1998) and during the specific arousal states of animals such as cats (Friauf & Baker, 1985) and monkeys (Kita, Chiken, Tachibana, & Nambu, 2007).

Immunohistochemical studies suggest that TTMNs have serotoninergic nerve endings (Thompson et al., 1998). Rouiller et al. (1986) reported on multisynaptic serotoninergic connections between raphe nuclei in the brainstem and TTMNs (Rouiller et al., 1986). The raphe nuclei are a cluster of cells located in the brainstem that contribute to the reticular formation, a higher brainstem center controlling behavior and arousal states through serotoninergic activity (Seikel et al., 2000; Siegel, Roeling, Gregg, & Kruk, 1999). The close relationship of the serotoninergic system and the MEM reflex supports the theory that tensor tympani contraction is triggered by non-auditory inputs. Although the efferent pathways from motoneurons to MEMs are well-characterized, fundamental unknowns continue to exist, which demand further query and research. Specifically, the multiple central circuits (reflex interneurons) that mediate the auditory and non-auditory responses of the MEMs are not fully understood.

6.5.3 SMN synaptic terminals

The diversity of inputs on SMNs is reflected in a variety of synaptic terminal types on these motoneurons (Brown et al., 2013). The ultrastructural features of synaptic terminals on retrogradely labeled SMNs in rat have been studied using electron microscopy (D. J. Lee et al., 2008; Brown et al., 2013**).** In SMNs, both the proximal

dendrites and SMN cell bodies were seen to be densely and evenly populated with synaptic terminals (D. J. Lee et al., 2008). A variety of inputs on SMNs include both excitatory and inhibitory properties. These terminals are classified into five major types according to the size and shape of their synaptic vesicles **(Figure 8**; D. J. Lee et al., 2008). The most common synaptic terminal type contained small, round vesicles **(Figures 8B & 8D**; D. J. Lee et al., 2008), and these are suggested to be excitatory (Uchizona, 1965). Similar terminals containing small round vesicles were observed on MOC neurons (Benson & Brown, 2006), and the source of these terminals were multipolar neurons residing in the CN. It is uncertain whether SMNs receive inputs from multipolar neurons or other neurons found in the CN (Benson & Brown, 2006). The second most common synaptic terminal type on SMNs contain large, round vesicles **(Figure 7A**; D. J. Lee et al., 2008). These large round terminals varied in size. A putative source of these inputs on SMNs is the globular bushy cell in the CN. Smith, Joris, Carney, and Yin (1991) described projections of globular bushy cells to the caudal brainstem near the facial motor nucleus where SMNs are located (Smith et al., 1991). In their study, large round vesicles were seen in terminals of globular bushy cells. The study by Lee (2008) revealed additional terminal types on SMNs **(Figure 7A).** The complex integration of inputs by SMNs reflected by the diversity of synaptic terminals seen in our ultrastructural study of SMN terminals may account for the multi-functionality of the MEM reflex (D. J. Lee et al., 2008).

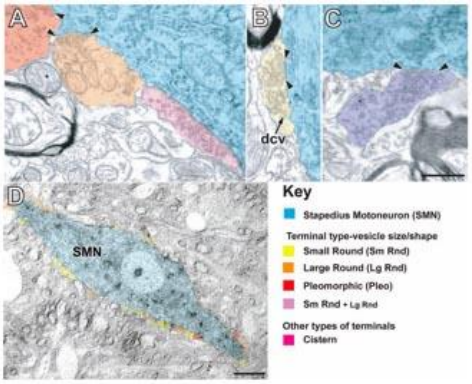

Figure 8. **Electron micrographs of the major terminal types seen on the stapedial motoneuron (SMN)** Note: Five types of synaptic terminals (see key) found on SMNs (cyan). Panel A: Synaptic terminals with pleomorphic vesicles (Pleo, red) and large, round vesicles (Lg Rnd, orange). Panel B: Synaptic terminal with small, round vesicles (Sm Rnd, yellow) and dense core vesicles (DCV). Panel C: Synaptic terminal with round vesicles of different sizes (Het Rnd, lavender). Panel D: Low magnification electron micrograph demonstrating the distribution of synaptic terminals on the SMN cell body and proximal dendrites. Synaptic terminals are color coded according to vesicle shape and size. The proportion of membrane in contact with synaptic terminals was approximately 50%. Scale bar = 0.5 mm (A-C), scale bar = 10 mm (D). Source: D. J. Lee et al. (2008). Reprinted with permission from the Association for Research in Otolaryngology.

6.5.4 TTMN synaptic terminals

Previous work from our laboratory has determined the types and richness of the synaptic inputs that fall onto the distal dendrites of retrogradely labeled rat TTMNs using electron microscopy (D. J. Lee et al., 2008; **Figure 9).** The frequency and morphology of terminal types observed on TTMNs were similar to that seen on SMNs (Benson et al., 2008; D. J. Lee et al., 2008). Synaptic terminals on TTMNs are classified into the four terminal types according to the size and shape of the synaptic vesicles. They were named (a) large round, (b) small round, (c) pleomorphic, and (d) heterogeneous. Compared with SMNs, there were slightly fewer small round terminal types found on TTMNs (D. J. Lee et al., 2008). SMNs also received a rare Cistern-type terminal that was not seen on TTMNs (D. J. Lee et al., 2008). In TTMNs, terminal types with round vesicles (large round and small round) constituted 40% to 45% (Conradi, Kellerth, Berthold, & Hammarberg, 1979). The relatively high proportion of terminal types containing round vesicles signifies that the TTMNs receive mainly excitatory input (Uchizona, 1965, Benson et al., 2013). The presence of pleomorphic terminal types **(Figure 9C)** suggests that TTMNs also receive some inhibitory input. As in the case of SMNs, the assignment of TTMN terminal types to the cell bodies of origin has not been established. The CN, SOC, serotoninergic sources, and higher cortex are all possible sources (Billig et al., 2007; Gelfand, 1984; Stach et al., 1984; Thompson et al., 1998).

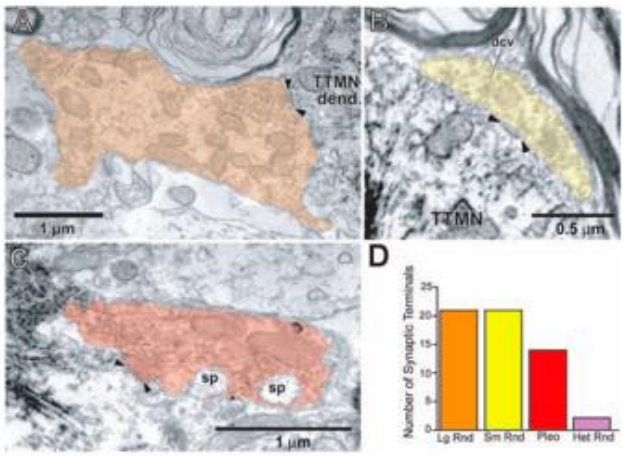

Figure 9. TTMN synaptic terminals
Electron micrographs of major round vesicle terminal types seen on tensor tympani motoneurons (TTMNs; D. J. Lee et al., 2008) in a rat model Note: Synapses are indicated with arrowheads. **Panel A:** Terminal with large round (Lg Rnd) vesicles. Myelin of axon is also indicated. **Panel B:** Terminal with small round (Sm Rnd) vesicles. A dense core vesicle (DCV) is seen occasionally. The round vesicles of Lg Rnd terminals are noticeably larger than the round vesicles of Sm Rnd terminals. Both of these terminal types have asymmetric synapses and are thought to be excitatory. **Panel C:** Terminal with pleomorphic (Pleo) vesicles. A third of terminals with pleomorphic vesicles engulf spines (SP) from the TTMN. Vesicles of

pleomorphic terminals have various shapes from flat to round. These terminals have symmetric synapses and are thought to be inhibitory. **Panel D:** Graph showing frequency of appearances of different synaptic terminals on TTMNs

6.6 CLINICAL APPLICATIONS

6.6.1 MEM reflex testing in diagnosis of middle ear and auditory brainstem disease

MEM reflex testing has been used to assist in the diagnosis of disorders of the middle ear (Jerger, Harford, & Clemis,1974, Biswas & Mal, 2013), cochlea (Olsen, Noffsinger, & Kurdziel, 1975), vestibulocochlear nerve (Anderson, Barr, & Wedenberg, 1969a), and brainstem (Jerger & Jerger, 1975). Measurements of the stapedial reflex can help to discriminate between otosclerosis and ossicular discontinuity (Anderson & Barr, 1971; Anderson, Jepsen, & Ratjen, 1962; Ebert, Zanation, & Buchman, 2008; Maurizi, Ottaviani, Paludetti, & Lungarott, 1985) and distinguish between cochlear and retrocochlear pathologies (Anderson, Barr, & Wedenberg, 1969b; Callan, Lasky, & Fowler, 1999; Chiveralls, Fitzsimmons, Beck, & Kernohan, 1976; Hunter, Ries, Schlauch, Levine, & Ward, 1999). The stapedial reflex can identify patients at risk of eighth cranial nerve tumors (Anderson et al., 1969b; Jerger & Hayes, 1983; Olsen et al., 1975), determine whether a facial nerve lesion is infra- or supra-stapedial (Djupesland, 1976; Fee, Dirks, & Morgan, 1975) or identify pathology of the central auditory system, such as an acoustic neuroma (Jerger, 1980; Jerger & Hayes, 1983; Jerger & Jerger, 1975; Jerger, Jerger, & Hall, 1979; Topolska & Hassmann-Poznanska, 2006).

Studies have explored the applicability of MEM reflex testing in the monitoring of pathophysiological changes in the auditory pathways that are associated with blunt head trauma (Nolle, Todt, Seidl, & Ernst, 2004) and industrial noise exposure (Zivic & Zivic, 2003). The MEM reflex is also being studied as a possible addition to the clinical investigations of non-auditory diseases, such as juvenile idiopathic arthritis (Ikiz, Unsal, Kirkim, Erdag, & Guneri, 2007), hydroencephalus (Counter, 2007), amyotrophic lateral sclerosis (Shimizu, Hayashida, Hayashi, Kato, & Tanabe, 1996), myasthenia gravis (Smith & Brezinova, 1991), atypical parkinsonian syndrome (Gironell et al., 2003), and myotonic dystrophy (Osanai, Kinoshita, & Hirose, 2001).

6.6.2 Interpretation of stapedius reflex abnormalities

In general, there are five main patterns to recognize when interpreting stapedial reflex abnormalities: (a) efferent, (b), afferent (c), central (brainstem), (d) unilateral (ipsilateral) and (e) global. The categorization of a specific pattern depends on the presence or absence of the ipsilateral and contralateral reflexes. In an efferent pattern, the stapedial reflex is abnormal in the recorded ear regardless of which ear is stimulated. It suggests a disruption of the efferent pathway on the same side of the recorded ear, for example caused by otitis media or a facial nerve abnormality that results in an inactive stapedius. In an afferent pattern, the stapedial reflex is abnormal in the stimulated ear regardless of which ear is being recorded. This pattern signifies a sensorineural hearing loss secondary to an acoustic neuroma affecting the afferent pathway. In a central

pathway (brainstem) pattern, all the crossed stapedial reflexes are reduced or absent. This phenomenon is commonly observed in disorders of the brainstem that interfere with the central auditory pathways. Central pathway patterns can also be seen in elderly patients with collapsed ear canals (Schow & Goldbaum, 1980). In the unilateral pattern, all the reflexes are abnormal except for the ipsilateral recording in one ear. This pattern occurs in a middle ear disorder with moderate conductive hearing loss in the recorded ear. It can also suggest a brainstem disorder severe enough to affect the crossed pathways but also the ipsilateral sensory pathway on the side of the recorded ear. The global pattern, in which all reflexes (ipsilateral and contralateral) are abnormal, reflects a severe to profound bilateral hearing loss, bilateral conductive hearing loss, or a central neural disorder affecting the crossed reflex pathways (Bess & Humes, 2008). A better understanding of the MEM reflex circuit diagram would help to localize a brainstem lesion associated with an abnormal reflex response.

6.6.3 Use of objective MEM reflex readings in cochlear implant programming

Electrically produced MEM reflexes have been used in the programming of speech processors in patients with cochlear implants. Recent years have seen a rapid expansion in the technology and general usage of both cochlear and auditory brainstem implantation devices. Monitoring an electrically produced MEM reflex allows the audiologist to objectively assess the integrity of the peripheral and central auditory brainstem pathways to facilitate the programming of young cochlear implant patients. For instance, measuring the loudness thresholds can be challenging in very young children. Electrically produced stapedial reflexes are therefore being investigated as an alternative to visual audiometric techniques in the programming of the speech processors in children with cochlear implants (Bordure, O'Donoghue, & Mason, 1996; Caner, Olgun, Gultekin, & Balaban, 2007). Advantages of monitoring an electrically produced stapedial reflex include providing more comfort to the child while ensuring a reliable replacement to behavioral audiometric techniques in assessing loudness thresholds (Caner et al., 2007; Hodges et al., 1997). Recent research has shown that the electrically elicited stapedius reflex may provide supportive information for improving programming of the cochlear implant in selected patients with inconsistent responses (Andrade et al., 2014).

6.6.4 Importance of MEM reflexes in ABI patients

Patients with neurofibromatosis-2 (NF-2) who have a nonviable auditory nerve because of either tumor infiltration or damage secondary to prior surgeries on the auditory nerve are ineligible for cochlear implants. These patients may be eligible for an auditory brainstem implant (ABI). An ABI is a surgically implanted electronic device that is suited for patients who are deaf secondary to a "retrocochlear" hearing impediment such as an illness such as a tumor destroying the cochlea or the auditory nerve such as in NF-2. The reduction or loss of hearing and speech recognition abilities in most NF2 patients is due to the tumor interrupting the blood supply to the cochlea, auditory nerve and/or to the cochlear nucleus. Also, direct physical compression by the tumor of the auditory nerve and the brainstem can impair neural excitability. The ABI was originally developed at the House Ear Institute in California in 1979 specifically for NF-2 patients

(House et al., 2001). These patients had lost function of the auditory nerves bilaterally following surgery to treat their vestibular schwannomas (Shannon et al., 1993). The way an ABI functions is similar to that off cochlear implants. Instead of utilizing direct electrical stimulation of the cochlea, as in cochlear implants, the ABI functions by way of direct electrical stimulation of the brainstem. The most recent ABI devices have 16 electrodes stimulating the brainstem. As per today, there are about 1500 patients with implanted ABI for various retrocochlear impairments (Herrman et al., 2015), the majority being due to tumors associated with NF-2 (Colletti et al., 2012). Other retrocochlear diseases that justify ABI surgery are auditory nerve aplasia and cochlear ossification syndromes (Colletti et al., 2003). The ABI has demonstrated benefits for some NF-2 patients in terms of optimizing sound awareness, improving the identification of environmental sounds and enhancing communication abilities when combined with lip-reading (Otto et al., 2002). Expanding the known neural framework of the auditory brainstem through transneuronal labeling of the middle ear muscle reflex is beneficial. It can, for example, improve the standards of ABI technology and additionally, enhance the surgical placement of implant devices. Observed middle ear muscles will theoretically provide an objective means or measure for helping the clinician/operator place the ABI electrodes onto the auditory brainstem. A better knowledge of the reflex pathways will thus improve surgical technique, minimize brainstem damage secondary to failed repeated attempts, improve signal processing/ device design, improve post operative hearing outcomes and ultimately enhance speech recognition. As rapid advances are being made in ABI technology where the feasibility of using infra-red light instead of electricity is under study (Verma et al., 2014), a more thorough understanding off the middle ear reflex pathways becomes all the more crucial. This will be addressed in more detail under “Discussions”.

6.7 CLINICAL SYNDROMES

Dysfunction of the tensor tympani or the stapedius muscle can result in a “middle ear myoclonus” syndrome (Badia et al., 1994). As per today, the syndrome is considered a clinical enigma due to the lack of consensus in our understanding of its etiology. This syndrome is characterized by isolated repeated involuntary contractions of either the tensor tympani or the stapedius muscle resulting in a high frequency tinnitus or “ear ringing/clicking” sounds (Bento et al., 1998). The tinnitus, which can be either objective or subjective, is resistant to different treatment methods (surgical or medical) and can be a source of tremendous distress to the sufferer. The etiology is as yet unclear though some have proposed that the disorder can stem from an imbalance in the neuro-physiological innervation of the muscular contracting units of the middle ear muscles as part of a systemic myoclonic disorder or as an isolated pathological entity (Bhimrao et al., 2012). Most reports of middle ear muscle myoclonus describes a tinnitus that varies greatly in quality (“buzzing” VS “pulsatile”), location (unilateral VS bilateral), intensity (i.e. loudness) and frequency (i.e. pitch) (Brosch et al., 2003). Diagnosis is suspected from the clinical history, “real time” observations of “synchronous movements of the

tympanic membrane with complaints of tinnitus and specific “saw tooth” patterns seen on long term tympanometry (Oliveira et al., 2003). Diagnosis is confirmed through direct visualization of the middle ear muscles via a tympanotomy surgical approach (Bhimrao et al., 2012). Visualization via a tympanotomy can also differentiate between myoclonus secondary to either stapedial or tensor tympani contractions (Cohen and Perez et al., 2003), in addition to ruling out other causes of tinnitus such as vascular anomalies and palatal myoclonus. Treatment ranges from supportive measurements (relaxation techniques), medication/ pharmacological intervention or surgery for recalcitrant cases. There is no consensus in pharmacological therapy and medications used have varied from topical application of botulinum toxin into the middle ear space (Liu et al., 2011), sedatives such as benzodiazepine (Zipfel et al., 2000), and skeletal muscle relaxants with anticholinergic properties (Ha CK, 2007). Ultimately, selective surgical resection (tenotomy) of either the stapedial tendon or the tensor tympani tendon (Hidaka et al., 2013) is the definite treatment with full resolution of symptoms. However, surgical intervention can risk damage to the facial nerve that traverses the middle ear cavity.

An analysis of the central neural circuitry governing the transport of impulses to and from the middle ear muscles, particularly the tensor tympani, will potentially allow us to further understand the pathological mechanisms behind this elusive syndrome. Though the role of the middle ear muscles, particularly the tensor tympani, is still not certain, there is a growing consensus that its function extends beyond the classical auditory function of its antagonist, namely the stapedius. Further study of the neural reflex pathways that innervate the two middle ear muscles can shed light on the neuro-physiological basis of myoclonus syndromes. This may spare future patients for surgical ablation of their middle ear muscles, and centrally acting pharmacological modalities can be adjusted accordingly based on the nature and/or localization of the neural disorder. By understanding the efferent component of the tensor tympani reflex pathway, the motor innervation of the reflex can be subdued either by medication or more specific transection procedures to eliminate excessive contraction of the middle ear muscles.

6.7 OLIVOCOCHLEAR REFLEX PATHWAY

6.7.1 Olivocochlear neurons

The medial olivocochlear (MOC) reflex pathway consists of olivocochlear (OC) neurons. These neurons form an efferent (motor) pathway through which the brain can control the cochlea. OC neurons have their cell bodies in the auditory brainstem's superior olivary complex. The superior olivary complex consists of two groups of neurons: 1) medial (M) OC neurons and 2) lateral (L) OC neurons. MOC neurons are large and are located mainly in the ventral nucleus of the trapezoid body (VNTB) **(Figure 10A).** LOC neurons are smaller and are located in and around the lateral superior olive (LSO) **(Figure 10B).** MOC neurons are activated in response to sound as part of the MOC reflex.

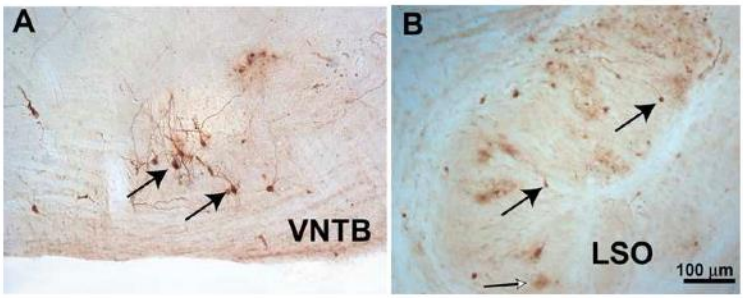

Figure 10. Photomicrographs of PRV-labeled OC neurons.
A: Labeled MOC neurons **(black arrows)** in the ventral nucleus of the trapezoid body (VNTB) on the side contralateral to the injected cochlea, in an animal that survived for 3 days after the injection. **B:** Labeled LOC neurons **(black arrows)** in the lateral superior olive (LSO) on the ipsilateral after a 3 day survival time (a different case from B). "Cloudy" areas of reaction product (one indicated by white arrow) may signify release of virus from PRV infected OC neurons.

6.7.2 Medial olivocochlear reflex

The MOC reflex performs a protective role in mammals similar to the MEM reflex. An activated MOC reflex helps in decreasing the effects of background noise and protects the cochlea from sound overstimulation (Ryugo et al. 2011). The function of LOC neurons is not as clear, but it is theorized that they may calibrate the neural sensitivity of the auditory nerves on the left and right sides (Darrow et al. 2006). MOC neurons receive inputs from the CN, which, as mentioned previously, is the first neural processing station in the auditory brainstem. Inputs from the CN determine their response to sound (Warr 1969; Thompson et al. 1991; Liberman and Guinan 1998; Ye et al. 2000; Brown et al. 2003). It has been postulated that OC (both MOC and LOC) neurons also receive inputs from higher levels of the auditory pathway like from the inferior colliculus (IC) (Faye-Lund 1986; Vetter et al. 1993) and the auditory cortex (Mulders and

Robertson 2000a). Studies have shown that similar to the theories concerning MEM reflex neurons, OC neurons can also receive input from areas in the brainstem that are not associated with the auditory pathway, such as inputs positive for noradrenalin (Mulders and Robertson 2005a) and serotonin (Thompson and Thompson 1995). The purpose behind the theorized regulation of the MOC reflex by non-auditory sources is uncertain but these sources probably regulate the sensitivity of hearing related to varying levels of concentration (Delano et al. 2007).

6.7.3 Identification of olivocochlear neurons

As with the MEM reflex pathway, the nature and identity of the neurons that provide inputs, auditory and non-auditory, to the OC neurons in the MOC reflex pathway is not fully known. Part of the research that forms the basis of this paper, explored and attempted to identify these inputs in an animal model using more effective viral tracers such as the Bartha strain of the pseudo-rabies virus (PRV). In most of the previous studies, the OC inputs have been recorded by using conventional neuronal tracers. Usually, one tracer had been used to retrogradely label the OC neurons and a second tracer was used to anterogradely label the suspected input. The main disadvantage of this technique was the need for large injection sites that made the interpretation of results difficult. For example, injections into the PVCN showed projections to MOC neurons (Thompson and Thompson 1991), but the reaction product at the injection site clouded the precise identity of the cells that made up the pathway. Transneuronal labeling using the PRV to label components of the MEM reflex pathway has avoided this problem (Enquist and Card 2003; Ekstrand et al. 2008). The PRV virus is absorbed by axons at the injection site and transported back to the neural cell bodies. Here the virus replicates and crosses to nerve terminals that synapse on the cells. The virus is later transported back to the cell bodies that provided those inputs. PRV has been used to study the OC neurons in the pioneering work of Horvath et al. 2003. After injections of PRV into the cochlea, labeling was first observed in the OC neurons and, at later times, within neurons in other locations. Transneuronally labeled neurons were found in the CN, IC, auditory cortex, and in centers not typically associated with the auditory pathway such as the subcoerulean nucleus and pontine dorsal raphe (Horvath et al. 2003). PRV was regarded as excellent for OC labeling because it is transported in the retrograde direction, therefore avoiding the complication of anterograde labeling of auditory nerve projections to the cochlear nucleus (CN) (Horvath et al. 2003).

The application of transneuronal labeling was accomplished to try to answer several important questions about the OC system. First, since cochlear injections of PRV label both MOC and LOC neurons, it was not clear whether transneuronal labeling in CN and IC goes via one or the other or both of these cell populations. The variable ratios of MOC and LOC neurons labeled from cases to case supported the theory that transneuronal labeling goes mainly via the MOC neurons. For example, one of the cases had almost entirely MOC labeling and this case had profuse transneuronal labeling. Overall analysis of all of the injections suggested a better relationship of MOC vs. LOC labeling with transneuronal labeling. Second, the aim was to identify the types of cells that were transneuronally labeled. This gave an idea as to the sources of inputs to the OC

neurons. In both CN and IC, the individual cell types have been previously well described in terms of the unique anatomy, physiology and dendritic projections (Osen, 1969; Oliver and Morest, 1984).

6.7.4 Intermediary interneurons

The CN has always been an interesting research topic because this nucleus performs a very important intermediate stage in both the MOC and the MEM reflex pathways (Guinan and Liberman, 1998; de Venecia et al. 2005). Previous studies have shown that CN transneuronal labeling is found in multipolar cells (Horvath et al. 2003), but the exact subtype within this heterogeneous class (reviewed by Doucet and Ryugo 2006) has not been identified. Recent work in mice using conventional tracers concluded that one class, called the "planar multipolar cells", projected to MOC neurons (Darrow et al. 2012). This thesis examined whether these "planar multipolar cells" and/or other subtypes could be identified via transneuronal labeling. It also examined whether the two types of IC neurons, cells with disc-shaped dendritic fields and cells of stellate shape, are transneuronally labeled. Finally, the research quoted in this paper confirmed the many types of "non-auditory" transneuronally labeled neurons reported by Horvath et al. (2003). However, it is still not known how these inputs participate in the OC reflex to sound. Current breakthrough in neurophysiology research using transneuronal tracers are shedding light on the information that may help us better understand the role of OC (and MEM reflex) neurons in human auditory and non-auditory processes.

6.8 REFLEX INTERNEURONS

6.8.1 Introducing reflex interneurons

As stated previously, one of the purposes of this thesis was to understand the components of the two descending auditory reflex pathways i.e. the MEM (more specifically, the tensor tympani reflex) and the olivocochlear reflex. This was achieved by identifying the motoneurons and the individual sources of modulatory brainstem inputs. In both pathways, the CN is an important structure because it is the first relay station for all ascending sound information originating in the ear. However, the identity and nature of the reflex or intermediary interneurons that exist within the CN is still not completely known. These "interneurons" in the CN are the cells that project either directly or indirectly to the efferent cells of either the MEM or olivocochlear reflex pathways.

6.8.2 The MEM reflex interneurons in the CN

The identity and organization of MEM reflex interneurons in the CN has been the focus of only a few studies (Billig et al., 2007; Borg, 1973; D. J. Lee et al., 2006; Lyon, 1978; Rouiller et al., 1986, 1989). The major anatomical subdivisions of the CN include the dorsal cochlear nucleus (DCN), anteroventral cochlear nucleus (AVCN), and

posteroventral cochlear nucleus (PVCN; Fitzgerald, Gruener, & Mtui, 2007; Palmer, 1987). Specific subtypes found in each subdivision of the CN display a variety of somatic and dendritic characteristics (Brawer, Morest, & Kane, 1974; Osen, 1969). For instance, the ventral cochlear nucleus (VCN) is known to contain spherical bushy cells, globular bushy cells, multi-polar cells, and octopus cells (Adams, 1983, 1986, 1989; Osen, 1965, 1969, 1970). The differences in morphology between cell types in the CN are associated with unique physiologic responses to auditory stimulation (Adams, 1986; Cohen, Brawer, & Morest, 1972; Evans & Nelson, 1973; Osen, 1969, 1970; Pfeiffer, 1966; Rhode et al., 1983) and projections to different targets in the auditory brainstem (Harrison & Warr, 1962; Schofield & Cant, 1996a; Van Noort, 1969).

6.8.3 Use of "distortion product otoacoustic emissions" to study reflex interneurons

Early anatomical studies have suggested that MEM reflex interneurons reside within the VCN (Borg, 1973). This was demonstrated by measuring the effects of surgical brainstem lesions on the acoustic impedance in rabbits (Borg, 1973). However, these lesions were nonspecific and may have damaged auditory nerve fibers of passage, confounding the results. In addition, these studies did not attempt to identity the reflex interneurons (Borg, 1973). A research group affiliated with our laboratory had used both anatomic and physiologic techniques in a rat model to localize reflex interneurons. The integrity of the MEM reflex pathway was assessed using a reflex metric (D. J. Lee et al., 2006). A reflex metric is a method of quantifying the strength of a physiologic response by subjecting an experimental model auditory system to a stimulus and then measuring the reflex response. In this study, the reflex assay was the suppression of sound produced "distortion product otoacoustic emissions" (DPOAE) in one ear in response to a reflex eliciting sound stimulus in the contralateral ear (Azeredo, Woods, Sterns, & Relkin, 2000; Relkin, Sterns, Schipper, Azeredo, & Woods, 2001; Smith, Sterns, Prieve, & Woods, 2005). The DPOAE is a measurement of sound ($2f1 - f2$) following the presentation of two fundamental frequency tones $f1$ and $f2$ (Kemp & Chum, 1980). The DPOAE requires normal forward and reverse acoustic transmission through the middle ear. Contraction of the MEMs reduces the stimulus and the emission associated with the DPOAE. The DPOAE was eliminated following the surgical transection of the MEMs, indicating that the assay was specific for the MEM reflex (Relkin et al., 2001).

6.8.4 The importance of acoustic striae for reflex interneurons

In the same experiments mentioned in 6.8.3 (D. J. Lee et al., 2006), a reflex assay was used to localize the MEM reflex interneurons in the CN. In the first set of animals, Lee et al, surgically transected the "acoustic striae" of the auditory brainstem ipsilateral to the sound stimulus (D. J. Lee et al., 2006). Acoustic striae are pathways in the auditory brainstem through which reflex interneurons are thought to exit the CN. The three striae are named in relation to their location as they exit the CN and are called "ventral," "intermediate," and "dorsal" (Borg, 1973; Held, 1893; Masterton & Granger, 1988; Moller, 1984).

6.8.5 Reflex interneurons exit through ventral striae

Sectioning of the dorsal and intermediate acoustic striae (the ventral acoustic striae was preserved) ipsilateral to the sound stimulus did not result in the elimination or the reduction of DPOAEs in the opposite ear (D. J. Lee et al., 2006). These findings suggest that the CN interneurons are likely to exit the CN through the ventral striae. Since the ventral striae are closely related to the VCN, the results support Borg's initial finding that the location of reflex interneurons is likely to be in the VCN (Borg, 1973; D. J. Lee et al., 2006). The predominance in the ventral striae of projections from globular bushy cells makes this cell type a possible candidate for a MEM reflex interneuron (Friauf & Ostwald, 1988; D. J. Lee et al., 2006; Smith et al., 1991).

6.8.6 MEM reflex strength dependence on the ventral cochlear nucleus

In another set of animals, the research group (Lee et al., 2006) lesioned different regions of the CN using the neurotoxic compound called Kainic acid. Kainic acid was selected because it destroys cell bodies but preserves the nerve fibers of passage (Coyle, Molliver, & Kuhar, 1978; McGeer & McGeer, 1978; Wuerthele, Lovell, Jones, & Moore, 1978). The DPOAE was measured in response to contralateral sound before and after lesioning of the DCN, PVCN, or AVCN. Post-experimental histology was examined and correlated with alterations in the MEM reflex strength (Bird, Gulley, Wenthold, & Fex, 1978; de Venecia, Liberman, Guinan, & Brown, 2005; Melcher & Kiang, 1996). Lesions of all subdivisions were associated with transient reductions in the DPOAE and therefore MEM reflex strength. A long-term reduction of the MEM reflex was seen in one case in which the injection was made in the VCN, and this correlated with neuronal loss in the PVCN, suggesting that the CN interneurons reside here (D. J. Lee et al., 2006). Additional data from our laboratory group (data not published) have shown that lesions of the VCN are associated with a reduction in MEM reflex strength. Interestingly, the VCN is also a region common to the MOC reflex pathways and acoustic startle reflex interneurons. The MEM, MOC, and startle reflexes are all triggered by sound and provide additional anti-masking effects to the auditory periphery (Liberman & Guinan, 1998).

6.9 TRANSNEURONAL LABELING OF REFLEX PATHWAYS

6.9.1 Pseudo-rabies virus (PRV)

Modified neurotropic viruses with decreased virulence have become powerful tracers for transneuronal labeling. Bartha pseudo-rabies virus (PRV) is an attenuated alpha herpesvirus that was originally developed for the pig industry as a vaccine against pseudo-rabies infection, but has a variety of properties that make it an ideal choice for studying the central auditory system as a transneuronal viral tracer. PRV has diminished cytopathogenicity, a broad host range, and demonstrates extensive staining of neuronal cell bodies and their dendrites (Card, 2001). Unlike conventional tracers, PRV can label chains of synaptically linked neurons by retrograde transport of viral particles

across synapses that link these neurons. Many studies have shown that PRV can invade axons and replicate, and then migrate in a retrograde fashion to transneuronally infect multisynaptic circuits in the brain (Boldogkoi, Bratincsak, & Fodor, 2002; Card, 2001; Helfferich, Uhereczky, Boldogkoi, Vitez, & Palkovits, 2003; Loewy, 1998; Sams, Jansen, Mettenleiter, & Loewy, 1995).

6.9.2 Direct Projections of CN reflex interneuron to Middle ear muscle Motoneurons

Billig et al. (2007) has used PRV to determine the location and identity of the tensor tympani reflex interneurons in the CN. Following injection of the tensor tympani muscle with PRV, bilateral transynaptically labeled neurons (mean neuronal counts: eight ipsilateral, five contralateral) in the dorsal and dorso-medial regions of the AVCN were seen (Billig et al., 2007; **Figures 11 & 12A).** Labeled neurons were also found in the dorsal PVCN, although to a lesser extent **(Figure 12B).** Based on both morphological appearance (large somas and orientation of dendrites that radiate across the CN; Doucet & Ryugo, 1997) and location in the AVCN, the majority of labeled neurons were classified as "radiate multipolar cells" (Billig et al., 2007). Radiate multipolar neurons have been previously shown to project to the dorsal cochlear nucleus (DCN) on the same side (Doucet & Ryugo, 1997; Schofield & Cant, 1996b) and are similar in appearance to the large commissural neurons that project to the contralateral CN (Cant, 1982; Doucet, Ross, Gillespie, & Ryugo, 1999; Schofield & Cant, 1996a; Wenthold, 1987). Billig et al. (2007) observed that the number of labeled neurons increased bilaterally in the AVCN at longer survival times (69 to 71 hours; means of 21 ipsilateral vs. 30 contralateral neurons). A few neurons were also detected in the PVCN and DCN, were centered mostly along the border between the two regions (Billig et al., 2007). These findings represent the first description of putative MEM reflex interneurons in the CN.

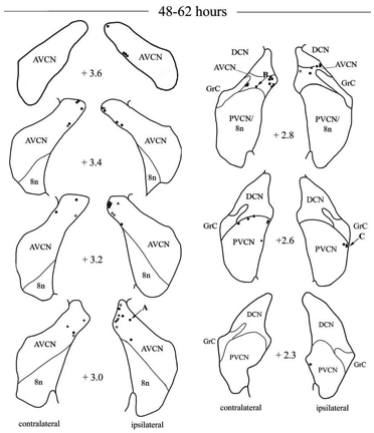

Figure 11. Camera Lucida drawings of ventral cochlear nucleus (VCN) labeling
Note: Brainstem atlas shows the locations of pseudo-rabies virus (PRV)–labeled neurons in the ventral cochlear nucleus (VCN) following injection into the right tensor tympani muscle. Survival times ranged from 48 to 62 hours. Each symbol represents a labeled neuron. Neurons on right side of each diagram are located ipsilateral to the PRV injection. The number between diagrams indicates the relative rostro-caudal distance in mm of the section from the obex. Arrows with A-C letters indicate the locations of infected neurons shown in Figure 13. 8n, vestibulocochlear nerve; AVCN, antero-lateral cochlear nucleus; DCN, dorsal cochlear nucleus; GrC, granular layer of cochlear nuclei; PVCN, posteroventral cochlear nucleus. Source: Billig et al. (2007). Reprinted with permission from *Brain Research*.

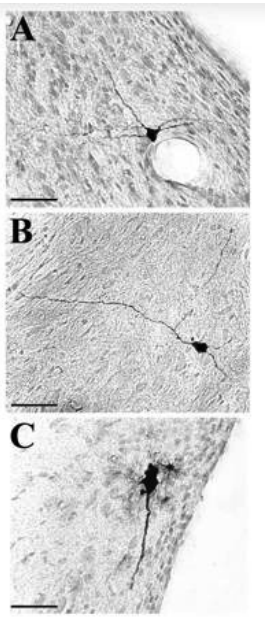

Figure 12. Pseudo-rabies virus (PRV)–labeled ventral cochlear nucleus (VCN) neurons following injection into the tensor tympani muscle
Note: **Panels A and B:** Radiate multipolar cells in the anteroventral cochlear nucleus (AVCN). **Panel C:** Large cell at junction of the posteroventral cochlear nucleus (PVCN) and the Schwann-cell border of the cochlear nerve. Scale bars represent 50 mm. Source: Billig et al. (2007). Reprinted with permission from *Brain Research*.

6.9.3 Location of CN reflex interneurons involved in MEM reflex pathway

A series of time-graded survival experiments by Windsor et al. (2007) demonstrated labeled CN interneurons 48 to 53, 62 to 72, and 96 to 120 hours following injection of PRV into rat stapedius and tensor tympani muscles. For stapedius injections, the majority of labeled neurons were seen bilaterally in the rostral half of the PVCN but also within regions clustered around the PVCN and DCN, with a mean neuronal count of four ipsilateral and five contralateral. Minimal labeling was seen bilaterally in the AVCN and auditory nerve root (Windsor et al., 2007). Tensor tympani muscle injections exhibited a different labeling pattern, in which CN neurons were seen bilaterally in the rostral half of the dorsal AVCN and interestingly, also around the SOC **(Figure 13;** Windsor et al., 2007) similar to the work of Billig et al. (2007) **(Table 1).** No labeling was seen in the PVCN, DCN, or auditory nerve root following tensor tympani muscle injections. The difference in locations of CN interneurons receiving impulses from the stapedius and tensor tympani muscles alludes to the possible existence of two separate groups of reflex interneurons in the CN involved in the MEM reflexes, namely, those in the PVCN controlling the stapedial reflex and those in the AVCN controlling the tensor tympani reflex. Observations by Billig et al. (2007) also strongly support the presence of direct

fibers from the VCN bilaterally to the TTMNs based on the temporal course of viral replication (Billig et al., 2007).

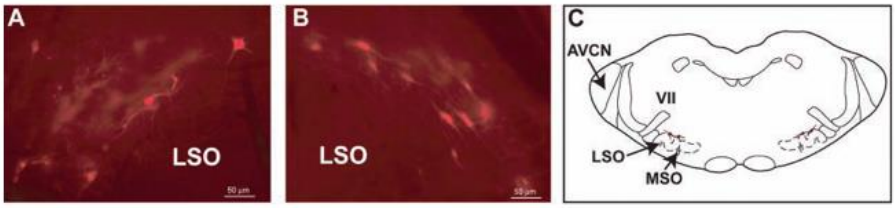

Figure 13. Bilateral superior olivary complex (SOC)
Bilateral superior olivary complex (SOC) labeling following pseudorabies virus (PRV) injection into the left tensor tympani (Windsor et al., 2007) Note: Fluorescence microscopy shows bilateral periolivary neurons (A: left, B: centre). Most of the PRV-labeled SOC neurons had two major dendrites arising from an elongated cell body, and were located immediately caudal to the region of labeled TTMNs (C: right). LSO = lateral superior olive; MSO = medial superior olive; AVCN = antero–ventral cochlear nucleus; VII = seventh cranial nerve.

Table1: Cases of injections of PRV into single tensor tympani muscles

Cases	Survival times (h)	Labeled motoneurons	Labeling in cochlear nuclei	Labeling in the superior olivary complex
TT 1	48		-	-
TT 2	48			-
TT 3	48			-
TT 4	62			-
TT 5	62			-
TT 6	62			-
TT 7	62			
TT 8	62			
TT 9	69			
TT 10	70			
TT 11	70			
TT 12	71			
TT 13	78			
TT 14	80			

Table 1. Cases are represented with their respective survival times. The shaded areas indicate the brain regions in the acoustic reflex pathways that contained labeling.

Consistent labeling was noted in the VCN (without SOC labeling) at survival times ranging between 48 and 62 hours, suggesting that the tensor tympani reflex may also consist of three types neuronal stations: *cochlea → VCN → motoneurons* (Billig et al., 2007). Direct connections between the CN and the motoneurons, without involvement of an additional synapse have been demonstrated, though the physiological relevance of a direct connection is still unclear (Billig et al., 2007; Borg, 1973; Itoh et al., 1987; Ito & Honjo, 1988; Van Noort, 1969). For instance, Itoh et al. (1987) provided evidence of direct afferent fibers projecting from the VCN to the TTMNs in cats after injecting a retrograde tracer into the pontine tegmentum, a region in the brainstem containing

TTMNs. Labeled neurons were observed bilaterally in the DCN and VCN (Itoh et al., 1987). Similar direct projections have been observed for other descending auditory pathways, such as the MOC reflex (Thompson & Thompson, 1991; Ye et al., 2000). It is not clear whether there is a similar direct projection of CN interneurons to stapedial motoneurons. Based on the tonotopical organization of the VCN (Saint Marie, Morest, & Brandon, 1989) and the cochleotopical organization of the auditory nerve fibers in the VCN (Fekete, Rouiller, Liberman, & Ryugo, 1984; Noda & Pirsig, 1974), the spatial arrangement of radiate multipolar CN interneurons controlling the tensor tympani could occupy the high frequency regions of the VCN (Fekete et al., 1984; Liberman,1991). The minimal labeling of neurons found in the auditory nerve supports the involvement of the tensor tympani muscle in the acoustic startle reflex (Y. Lee, Lopez, Meloni, & Davis, 1996). Projections of CN interneurons directly onto the MEM reflex motoneurons have been described as being similar to those seen in the MOC pathway (Thompson & Thompson, 1991; Ye et al., 2000).

6.9.4 Indirect Projections of CN reflex Interneuron to Middle Ear Muscle Motoneurons

Studies using transneuronal tracers (Rouiller et al., 1986, 1989) have revealed an additional synapse in the LOC & MOC neurons at the level of the SOC in the mediotrapezoid region **(Figures 13A & B)**. In a similar experiment, transneuronally infected neurons appeared close to the SOC bilaterally after injection of a herpes viral tracer into the tensor tympani muscle of rats (Rouiller et al., 1986). These findings suggest that some CN interneurons may project indirectly to MEM motoneurons by way of the SOC. However, labeled neurons in these early viral studies did not represent the entire network of neurons involved in the MEM reflex pathway as not all the known CN interneurons were labeled in these experiments. Billig et al. (2007) detected a small number of labeled cells bilaterally in the MNTB of the SOC as early as 62 hours following the injection of PRV into the rat tensor tympani**.** There was a clear ipsilateral dominance, with some labeling also seen in the periolivary (PO) cell groups. More obvious labeling in both the MNTB and the dorsal, medio-ventral, and lateral aspects of the PO cell groups was observed following inoculation times between 69 and 71 hours. After 69 hours, Billig et al. (2007) reported a mean of 38 ipsilateral labeled neurons compared with 18 contralateral labeled neurons in the SOC. After a survival time of 78 to 80 hours, the number of labeled neurons in the SOC increased to a mean of 462 ipsilateral versus 246 contralateral neurons (Billig et al., 2007). On the basis of their morphology, the labeled neurons were characterized as “marginal cells,” “microneurons”, and other cell types that could not be classified (Billig et al., 2007). The presence of these cell types in the SOC was deemed significant because prior studies have shown that marginal cells in the VCN project to the PO cell groups and the contralateral MNTB of the SOC (Friauf & Ostwald, 1988). Additionally, neurons originating in the PO and MNTB have also been shown to project to the TTMNs on both sides in rabbit (Borg, 1973) and rat (Rouiller et al., 1986). Preliminary results from PRV injections into rat stapedius and tensor tympani muscle (Windsor et al., 2007) showed transneuronally labeled neurons in the bilateral PO cell groups dorsal and medial to the lateral superior olive (LSO) 48, 62, and 96 hours following an injection but not to the extent and increasing pattern as described by Billig et al. (2007; **Figure 13).**

6.9.5 Transneuronal involvement of OC neurons in MEM reflex pathway

Involvement of the SOC suggests that one possible MEM reflex circuit diagram would include four stations or elements: cochlea (spiral ganglion cell) → VCN (reflex interneuron) → SOC (superior olivary complex) → middle ear muscle motoneuron. Since the SOC is a relay station for ascending and descending auditory information traveling to and from both ears, some nerve fibers leaving the CN send collaterals to the SOC before forming the lateral lemniscus. The lateral lemniscus constitutes a specialized tract of axons that carries acoustic information from the CN to various brainstem nuclei and the midbrain (Kelly, van Adel, & Ito, 2009; Saldana, Aparicio, Fuentes-Santamaria, & Berrebi, 2009). It is located medial to the CN in the caudal portion of the pons and consists of three main nuclei, namely the MSO, the LSO, and the MNTB. The MSO receives inputs from the CN and is sensitive to inter-aural time differences. The LSO also receives inputs the CN and responds exclusively to inter-aural amplitude differences (Fisher & Harrison, 1962; Harrison & Warr, 1962; Oliver & Beckius, 1996; Ollo & Schwartz, 1979).

Through the information processed by both nuclei, the SOC collectively assists in sound localization by analyzing time and amplitude differences between acoustic signals. There are in addition between six and nine PO cell groups interspersed around the three main nuclei. The SOC plays an as yet undefined role in the MEM reflex pathway. Early mechanical degenerative studies demonstrated that CN reflex interneurons synapse onto third-order neurons in the SOC on the same and opposite side of the stimulated ear (Borg, 1972, 1973). This was based on surgical lesions made in the medial part of the SOC that affected both the contralateral and the ipsilateral sound-produced MEM reflex in rabbit (Borg, 1972, 1973; Moller, 1984). There are possibly coexisting indirect pathways (Moller, 1984; Shlomo & Silverman, 1991; Wiley & Block, 1984; Wilson, Shanks, & Lilly, 1984) that are multisynaptic and project in parallel to the main MEM reflex arc. These pathways are largely unidentified and are likely to involve the extra-pyramidal system (Courville, 1966). Anatomical degenerative studies have demonstrated that when lesions of the reticular formation are made rostral to the SOC, there is degeneration in the facial motor nuclei (Borg, 1973). Compared with the direct pathways, the indirect pathways are slower and more sensitive to medications such as barbiturates, and are also dependent on the arousal state (Baust, Berlucchi, & Moruzzi, 1964; Borg & Moller, 1975; Salomon, 1963). The fact that several pathways are involved in the MEM reflex may explain the multi-functionality of the tensor tympani and stapedius muscles in response to a wide array of auditory and non-auditory stimulation.

7. STUDY AIMS

This thesis is in essence an amalgamation of several related studies. The main objective of these studies is to discover new characteristics of the middle ear muscle (particularly the tensor tympani) reflex pathway and the olivocochlear pathway. It aims to present evidence at a cellular level that sheds light on the multifaceted function of the two reflex pathways in mammals. It also aims to locate possible sources for both auditory and non-auditory neuronal input. To fulfill the greater objective, smaller goals needed to be satisfied and these included the analysis of labeled (chemical or viral) neurons and the postulated sources of their modulating input (auditory and non auditory). Historical insight was expanded in a parallel study to provide depth behind the relevance of these new cellular findings by relating them to the first theories made on the function of the middle ear muscle by the famous 16th Century Italian scientist, Hieronymous Fabricius (1533-1619). Although not directly related to science, it is important to mention a few insights relating to the works of Hieronymous Fabricius who was a pioneer in approaching anatomy from a structure-function relationship. Fabricius' writings in his book "De Visione, Voce et Auditu", which I had the pleasure of translating and analyzing, provided the foundation for contemporary theories on the role of the tensor tympani because he proposed those ideas during an era when great strides were being made to increase our understanding of ear anatomy and physiology. A list of aims are listed below:

<u>Aims for Study 1 (basic science) Tensor tympani reflex pathway:</u>

i) To determine the location of tensor tympani motoneurons (TTMN) in the auditory brainstem and their relation to the trigeminal motor nucleus.
ii) To distinguish between multiple tensor tympani motoneurons subtypes.
iii) Conducting a morphometric analysis of TTMN dendrites (structure, localization, length and branching units) to determine their potentiality for receiving and spreading impulses to and from other regions of the brainstem.

<u>Aims for Study 2 (basic science): Olivocochlear reflex pathway:</u>

i) To identify MOC & LOC neurons using PRV as the labeling technique.
ii) To make macroscopic comparisons between PRV-labeled MOC & LOC neurons.
iii) To assess how the extent of labeling of OC neurons changes with survival time after the primary injection.
iv) Analyzing and characterizing PRV labeled non-olivocochlear structures.
v) Studying the spread of labeling in other regions of the brainstem with changing survival times.
vi) Studying the characteristics and subtypes of PRV labeled cells in the cochlear nucleus.
vii) Analyzing the presence and morphology of PRV labeled cells in higher neuronal

centers (such as the inferior colliculus and the auditory cortex).

Aims for Study 3 (historical & analytical): History of tensor tympani:

i) Understanding the translated chapter on tensor tympani in "De Visione, Voce et Auditu (1600).
ii) Studying the theories Fabricius published on tensor tympani anatomy.
iii) Studying the theories Fabricius published on middle ear muscle function.
iv) Describing Fabricius' theories on the possible non-auditory role of middle ear muscles.

8. METHODOLOGY

8.1 TENSOR TYMPANI (FLOUROGOLD) EXPERIMENT

A total of 18 mice were used for this experiment. The CBA/CaJ mouse strain was used because of their excellent hearing thresholds (Yoshida et al., 2000). The mice were 3–6 months old and weighed 25–35 g. All experimental procedures on animals were performed in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals and were performed under approved protocols at the Massachusetts Eye and Ear Infirmary. Mice were anesthetized with Ketamine (100 mg/kg) and Xylazine (20 mg/kg). Boosters of Ketamine and Xylazine were administered as needed. The level of anesthesia was monitored by paw–pinch withdrawal reflex and response to surgical manipulation. In order to label TTMNs, a left post auricular surgical approach with partial removal of the bulla was used for exposure of the middle ear. Hydroxystilbamidine (Fluorogold (FG); Fluorochrome, Denver, CO) was injected into the muscle belly of the left TT. Fifteen injections were made with glass pipettes (inner diameter 0.6 mm) filled with 1–2 μl FG. The tip of the glass pipette was inserted into the muscle and remained there for 1–2 min to allow for passive infiltration of FG into the TT. To test a different method of injection, another 3 mice were injected with the 10 μl Hamilton syringe (inner diameter 0.33 mm) filled with 1 μl FG. The tip of the syringe was inserted through the muscular sheath and into the medial belly of the TT muscle. The FG was then pressure injected slowly and the muscle was seen to swell with the administration of FG. The numbers of labeled TTMNs from the syringe-injected cases was somewhat lower than the pipette cases, but this difference was not statistically significant (t-test; t=0.219). Dental pledgets were used to minimize secondary spread into the surrounding structures of the middle ear. Following surgical closure and recovery, the animals underwent a 7–9 day survival period prior to perfusion. At the end of the survival period, the animals were re-anesthetized and perfused using trans-cardiac method with physiological saline followed by 4% para-formaldehyde in 0.1 M sodium phosphate buffer (pH 7.2). After post fixing for approximately 1 h, brainstems were dissected and blocked immediately rostral to the inferior colliculus and caudal to the cochlear nuclei. A pin was used to mark the right side of the dissected brainstem. The brainstem was placed in fixative solution for another 1 h, before it was put into 30% sucrose for 1–2 days. The following day, transverse sections of 80 μm thickness were cut on a freezing microtome and placed into a phosphate buffered saline (PBS: pH 7.4). All sections were collected from a point caudal to the dorsal cochlear nucleus extending to just rostral to the inferior colliculus. Sections were incubated with 1:100,000 anti-FG rabbit IgG fraction (Fluorochrome, Denver, CO) for 24 h. Sections were then incubated in 1:1000 biotinylated goat anti-rabbit secondary antibody solution (Vector Labs). Standard immuno-histochemistry technique using diaminobenzidine (DAB) solution was used to visualize reaction product. Morphological measurements: A light microscope fitted with a Camera Lucida was used to identify and draw the labeled neurons. Images were acquired using a Hamamatsu CCD (Charged-Coupled Device) digital camera. A labeled

neuron was counted if the cell body was judged entirely or for the most part within that section, and every labeled neuron was counted. No correction was made for the possibility of counting the same labeled neuron multiple times in adjacent sections. Labeled neurons were traced onto paper using the camera Lucida tube on a light microscope with 40 degree objectives. The major axis diameter was measured along the longest axis of the labeled cell body. The minor axis diameter was measured as a line perpendicular to the major axis of the labeled cell body. The sample of dendrites was composed of dendrites that were not intertwined with other labeled elements. Dendrites that split into two processes within 10 μm from the soma were considered as two separate dendrites. Dendrites were not counted if their reaction product became very light towards the ends of the dendrite, giving the dendritic tips a faded appearance. Dendrites were not included if they became entangled with other dendrites or exited the plane of section. The reconstructed dendrites were traced onto paper and the dendritic angle was measured using a protractor by drawing a line from soma to dendrite tip, using the angle of this line with respect to the dorsal direction. For all sections, the dorsal/ ventral direction was defined as parallel to the midline of the section. Dendrite length was taken as the length of the entire course of the dendrite from soma to tip measured using a flexible contour against a ruler. Statistical tests were conducted, and the 5% level used as a measure of significance.

8.2 OLIVOCOCHLEAR (PSEUDO RABIES VIRUS) EXPERIMENT

All the surgical procedures and techniques for anesthesia associated with the PRV study complied with the NIH (National Institute for Health), Harvard Medical School Biosafety Committee, and Massachusetts Eye and Ear Infirmary guidelines and were performed in a Biosafety Level 2 suite. Adult guinea pigs (500 to 800 g) were anesthetized with pentobarbital (19 mg/kg, i.p.), droperidol (10 mg/kg, i.m.), and fentanyl (0.2 mg/kg, i.m.). A post-auricular surgical approach was performed and the bulla opened to visualize the basal turn of the left cochlea. The round window membrane was torn and the stapes footplate was displaced. PRV 152, which is isogenic with PRV-Bartha but contains an GFP (green fluorescent protein) expression cassette cloned into the middle of the PRV IgG gene and grown in pig kidney cells (PK15) as described previously (Smith et al. 2000; Pickard et al. 2002), was provided by Dr. L.W. Enquist (Princeton University). The virus was stored at −80 °C and frozen aliquots were quickly thawed to 37 °C in a water bath, ultrasonicated (Misonix, Inc.), and centrifuged before inoculation. Aliquots contained 109 plaque-forming units per milliliter. Injections of PRV were made through the round window in steps of 2 μl with a 10- μl Hamilton syringe. Fluid was wicked from the oval window between steps. An average total of 15 μl (range of 5–30 μl) was injected. Dental pledgets were used to minimize secondary spread into surrounding structures of the middle ear. Following surgical closure of the wound and recovery, the animals underwent a 1–5-day survival time in the Biosafety Level 2 suite. The animals were then re-anesthetized and perfused through the vascular system with saline and then with 4 % para-formaldehyde in 0.1 M phosphate buffer (PB) without salt (not to be confused with PBS which is phosphate buffer saline: both PB and PBS have a pH of 7.4). Dissected brainstems or cortex pieces were immersion- fixed for 2–4 h and then washed in PBS. For most animals, the brainstem was then immersed in 30 % sucrose overnight

and then sectioned (80 μm) on a freezing microtome. Brainstem sections were collected that included the CN, superior olivary complex, and the IC. In some cases, pieces of the temporal cortex were embedded in a gelatin albumin mixture and sectioned (also at 80 μm) on a Vibratome. In two animals, the injected cochleas were decalcified and then embedded according to the method of Hurley et al. (2003), and sections cut on a freezing microtome. In a few animals, sections were examined in the fluorescent microscope without further processing. In most animals, sections were blocked for 1 h with 10% NGS (normal goat serum) /1% BSA (bovine serum albumin) solution, followed by incubation with 1:500 primary antibody anti-GFP rabbit IgG fraction (Invitrogen Inc.) for 2 days at 4 °C to penetrate to the interior of the section. The sections were washed extensively in and incubated with 1:500 biotinylated goat anti-rabbit secondary antibody (Invitrogen Inc.) for 2 h, washed again, and then incubated with an ABC (Avidin Biotin Complex) kit and finally with diaminobenzidine (DAB). Sections were mounted and cover slipped for viewing in the light microscope. Although the reaction product is formed to GFP, we described neurons with the reaction product as "PRV-labeled." A total of 33 guinea pigs received cochlear injections and underwent brainstem tissue processing. Six of the cases had no labeled OC neurons and no labeling elsewhere in the brainstem. Twenty-seven cases had labeled OC neurons **(Table 2).** The cortices were processed in 13 of these animals. Two additional guinea pigs received control injections of PRV into the middle ear space (instead of the cochlea). After a 3-day survival time, there were no labeled neurons in the brainstem of these two animals. Three other guinea pigs received cochlear injections of 30 % horseradish peroxidase in saline. After a 24-h survival time, they were perfused and sectioned as described above. Sections were reacted with tetramethylbenzidine (Mesulam 1982) and counterstained with neutral red. Labeled neurons were counted only if they contained a dark reaction product in comparison to background. From plots of the numbers of labeled neurons in different brainstem centers vs. the number of labeled OC neurons, linear fits and linear (Pearson) correlation coefficients (R) were computed using Kaleidagraph software. Correlation coefficients near 1 represent a linear relationship in which the variables are highly correlated (Pagano and Gauvreau 2000). The relationships were considered significant if the P values were ≤ 0.05. Internal subdivisions of the nuclei were delineated as described in previous works on the rodent CN (Hackney et al. 1990) and the IC (Oliver and Morest 1984; Loftus et al. 2008). Micrographs were obtained with a compound microscope fitted with a digital camera and were not further processed.

Table 2

PRV labeling data

GP	PRV (µl)	Survival time (days	Number of PRV-labeled neurons *		
			LOC	MOC	CN
29	25	1	1	0	0
30	25	1	0	4	0
1	25	2	5	7	0
2	20	2	1	0	0
10	20	2	1	0	0
15	10	2	1	1	0
16	10	2	6	43	1
17	8	2	51	0	0
19	10	2	4	0	0
11	20	2	1	1	0
26	25	3	7	10	33
2008_7	6	3	4	4	8
8	5	3	63	33	59
12	20	3	114	214	175
14	30	3	255	384	283
21	10	3	7	6	2
22	10	3	48	10	62
24	18	3	39	21	92
28	25	3	134	172	396
2008_1	24	3	72	137	110
7	5	4	39	233	185
5	5	4	2	65	51
6	5	4	31	466	500
2008_6	18	4	6	3	20
2008_7	12	4	28	8	5
3	20	5	42	220	228
4	20	5	46	96	63

* Counts are of bilateral labeling

Table 2. PRV labeling data in guinea pigs and neuron count with corresponding survival time (days).

8.3 FABRICIUS (HISTORICAL) STUDY

The design of the historical study involved first a review off the literature concerning the life, background and scientific contributions of Hieronymous Fabricius (1533-1619) using various sources. These sources were attained from the collections of various libraries associated with Harvard University (Cambridge, MA, USA). We also analyzed the translated Latin text that focused on the middle ear muscles from a first edition of Fabricius' book entitled De Visione, Voce et Auditu (The Vision, Voice and Hearing, 1600). This book was available for study at the Center for the History of Medicine, Countway Library, Harvard Medical School.

9. SUMMARY OF RESULTS

9.1 **STUDY 1:** NATURE OF LABELLED COMPONENTS OF THE TENSOR TYMPANI MUSCLE REFLEX PATHWAY AND POSSIBLE NON- AUDITORY NEURONAL INPUTS

9.1.1 Retrogradely labeled TTMNs were distributed ventro-lateral to the ipsilateral trigeminal motor nucleus

Fluorogold (FG)-labeled TTMNs were stained a dark brown color after histological processing. The labeled cells were located as a narrow curvi-linear column in an area ventro-lateral to the ipsilateral trigeminal motor nucleus **(Figure 14).** In all 18 cases, the dorsal (superior) horn of the column curved more than the ventral (inferior) horn. The column began to appeared close to the rostral pole of the facial motor nucleus and extended up towards the level of the inferior colliculus. The labeled TTMNs were found on the left side of the brainstem, ipsilateral to the injection site. In 13 of the 18 cases, incidental or "extra" labeling of non-TTMN structures was found caudal to the trigeminal motor nucleus, within the ipsilateral facial motor nucleus, the inferior salivatory nucleus and the parvicellular reticular tract.

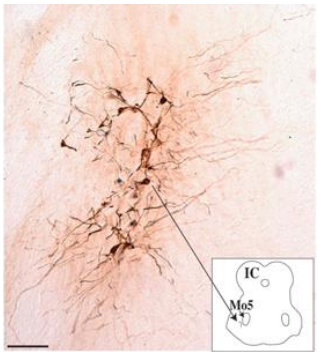

Figure 14. Micrograph of representative FG-labeled TTMNs (Mukerji et al., 2008)
Labeled TTMNs in mice were organized as a curvi-linear column ventro-lateral to the trigeminal motor nucleus: Mo5 (see inset). IC: Inferior Colliculus. Scale bar: 100 μm.

9.1.2 There were three observed TTMN subtypes: stellate, octopus-like and fusiform

Labeled TTMNs exhibit different morphologies **(Figure 15A).** Each TTMN was categorized into one of three subtypes, based on the number and orientation of the

primary dendrites that projected from the cell body. In our study, the following criteria and names were used; *"Stellate"* (star shaped) TTMNs had three or more dendrites leaving the cell body. *"Octopus-like"* TTMNs had two dendrites projecting from one side of the cell body. *"Fusiform"* TTMNs had two dendrites projecting from opposing poles of the cell body. The distinction between the "octopus-like*"* and "fusiform" subtypes was usually clear but I acknowledged the presence of a small number of intermediates that fell between the criteria described above. "Stellate" TTMNs were however very distinct from the other subtypes. The average number of primary dendrites originating from the cell body of a "stellate" TTMN was 3.5 (n=30 "stellate" TTMNs). The highest number of primary dendrites that projected from a cell body was 5. From our total sample of 945 classified TTMNs, "octopus-like" TTMNs were the most common (52% of the total sample) **(Figure 15B: left graph)**, followed by "fusiform" (35%) **(Figure 15B: right graph).** and then lastly "stellate" (13%) **(Figure 15B: middle graph).** The average minor axis diameter of 334 labeled TTMNs was 13.0 μm, and the average major axis diameter of 258 labeled TTMNs was 23.5 μm. The minor and major axis average sizes are plotted according to subtype in **Figure 15B: middle graph**. There was no statistical difference in the cell size (one way ANOVA; F=5.469, p=0.005) between TTMN subtypes. The average minor axis diameter of "octopus-like" shaped TTMNs (15.5 μm; n=53 octopus-like TTMNs) was only slightly larger than the average minor axis diameters of both "fusiform" (12.7 μm; n=53 "fusiform" TTMNs) and "stellate" TTMNs (13.0 μm; n=20 "stellate" TTMNs). The average major axis diameter of "octopus-like" TTMNs (24 μm; n=122 "octopus-like" TTMNs) was similar to the average major axis diameters of "fusiform" (23.6 μm; n=102 "fusiform" TTMNs) and only slightly larger than the average major axis diameter of stellate TTMNs (22.8 μm; n=34 TTMNs). Plots of major vs. minor axis did not separate the TTMNs into subtypes (data not shown). There was also no clear relationship between a TTMN subtype and its position along the rostro-caudal axis of labeled TTMNs. Instead, the three TTMN subtypes were distributed evenly within the populations of TTMNs throughout the sections.

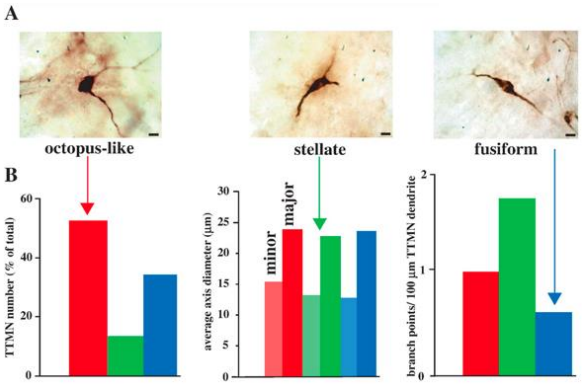

Figure 15. Tensor tympani motoneuron subtypes.
(A) Micrographs of the three TTMN sub-types; "octopus-like", "fusiform" and "stellate". Scale bars: 10 µm. **(B)** Left graph shows the percentage of each TTMN subtype in 729 labeled TTMNs from 14 cases; 52% were octopus-like (red bar), 7% were stellate (green bar) and 41% were fusiform (blue bar). Middle graph shows the average minor axis diameter (light color) and major axis diameter (dark color) of each TTMN subtype; octopus-like: 15.0 µm (n=50) and 24.0 µm (n=122), stellate: 13.5 µm (n=20) and 22.8 µm (n=34), fusiform: 12.6 µm (n=50) and 23.6 µm (n=102). Right graph shows the average number of branch points in the first 100 µm of primary dendritic length for each TTMN subtype; octopus-like: 0.98 (n=50), stellate: 1.67 (n=50) and fusiform: 0.6 (n=20).

9.1.3 TTMN dendrites demonstrated a particular structure and orientation

The labeled TTMN dendrites were long, radiating and minimally branched **(Figure 16).** The dendrites never entered the borders of the trigeminal motor nucleus, the main efferent center of the fifth cranial nerve, instead touching only the most lateral and dorsal edges. I re-constructed 57 dendrites from 18 cases across all three TTMN subtypes ("octopus-like", "fusiform" and "stellate") that could be measured from the cell body all the way to the tip without fading. The lengths were then plotted as a function of their orientation in polar format **(Figure 17).** The lengths of the 57 reconstructed TTMN dendrites ranged from 154 µm to 650 µm, with an average of 362 µm. Most of the reconstructed dendrites traveled dorso-medially (n=37) compared to the other quadrants; ventro-medial (n=7), ventro-lateral (n=8) and dorso-lateral (n=5). The number of branch points of the primary dendrite varied with TTMN subtype. In 120 labeled TTMN dendrites, the average number of branch points within the first 100 µm of primary dendrite length was counted. "Stellate" TTMN dendrites branched the most with an average of 1.67 branch points (n=50 "stellate" TTMN dendrites) per 100 µm of primary dendritic length. "Fusiform" TTMN dendrites exhibited the least branching with an average of 0.6 branch points (n=20 "fusiform" TTMN dendrites). "Octopus-like" TTMN dendrites had 0.98 branch points (n=50 "octopus-like" TTMN dendrites).

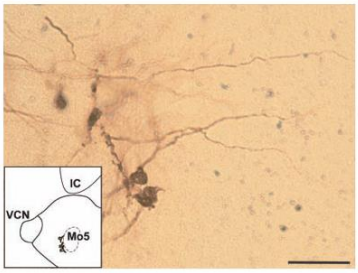

Figure 16. Fluorogold labeled TTMNs with dendrites.
FG-labeled TTMNs with dendrites extending medially beyond the pool of labeled TTMNs located ventro-laterally to the trigeminal motor nucleus (see inset: bottom left corner). These TTMNs were located on the left side of the brainstem so the medial direction is toward the right. This section is located along the rostral–caudal distribution of labeled TTMNs at the position about 1/3 from the caudal end. Micrograph is a montage of two images taken of a single section at different focal planes. Scale bar: 50 μm. *(Mukerji et al., 2008).*

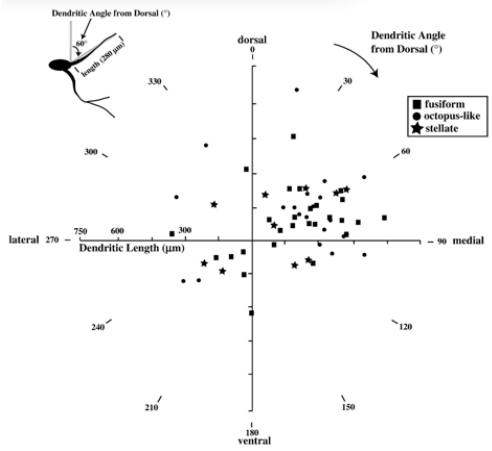

Figure 17. TTMN sub-types and dendritic orientation.
Polar plot showing the lengths (μm) and dendritic angles (°) of 58 reconstructed FG-labeled dendrites across the three TTMN subtypes; octopus-like (circles), stellate (stars) and fusiform (squares). Dendrites directed dorso-medially were more numerous and were the longest in length. Inset shows measurement of dendritic angle by drawing a line from soma to dendrite tip and measuring the angle of this line with respect to the dorsal direction.

9.2 **STUDY 2:** NATURE OF PRV LABELED COMPONENTS OF THE OLIVOCOCHLEAR PATHWAY AND THE IDENTIFICATION OF OTHER NEURONAL INPUTS

9.2.1 Identification of PRV-labeled MOC & LOC neurons

Following injections of PRV into the cochlea, labeled neurons were found in the superior olivary complex. Labeled neurons of small size were found within the lateral superior olive (LSO, **Figure 18A**), with a few of larger size on its margins. These neurons were grouped together as LOC neurons. MOC neurons were found in the ventral nucleus of the trapezoid body (VNTB, **Figure 18B**) and dorso-medial periolivary nucleus (DMPO). These neurons were well filled with reaction product that often extended into the dendrites. Their appearance was generally similar to such neurons labeled by conventional neural tracers such as horseradish peroxidase (HRP) in previous studies (Strutz and Bielenberg 1984). One difference was that sometimes in the vicinity of PRV-labeled neurons, there were "cloudy" extracellular deposits of reaction product (**Figure 18A**) and labeled profiles ("blebs," in **figure 18B**) that may signify cells in the late stages of cytopathic changes from PRV infection (Billig et al. 2007).

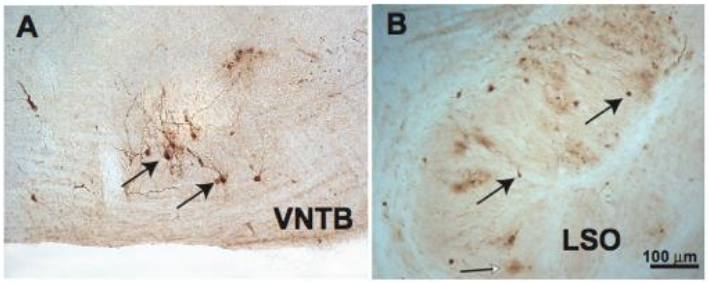

Figure 18. Photomicrographs of PRV-labeled neurons
A: Labeled LOC neurons (black arrows) in the lateral limb of the lateral superior olive (LSO) on the side ipsilateral to the injected cochlea after a 3-day survival time. "Cloudy" areas of reaction product (white arrow) were sometimes observed in areas of PRV labeling (see text). This section is at the mid-point of the LSO's rostro-caudal extent. **B**: Labeled MOC neurons **(black arrows)** in the ventral nucleus of the trapezoid body (VNTB) on the side contralateral to the injected cochlea, in an animal that survived for 3 days after the injection (a different case from A). A labeled group of "blebs" is indicated by a white arrow (see text). This section is from the caudal VNTB, near the caudal tip of the LSO.

9.2.2 Morphometric comparison of PRV-labeled MOC & LOC neurons

MOC somata major and minor axis diameters (average 25.4°—15.4 µm, SD 5.4°—3.3 µm, for 51 neurons) were significantly larger than LOC somata from within the LSO (average 15.9°—10.5 µm, SD 3.1°—2.6 µm, for 28 neurons). The few neurons

located on the LSO margins (LOC "shell neurons," Vetter and Mugnaini 1992) were large (average 28.9°—11.9 μm, SD 5.2°—2.2 μm, for four neurons). Some MOC neurons and LOC shell neurons had dendrites with visible spines (Mulders and Robertson 2000b; Benson and Brown 2006; Brown et al. 2013a). Axonal labeling was uncommon, but several cases had a few OC axons projecting dorsally in the brainstem.

9.2.3 Pattern of PRV labeling and survival time after injection

The pattern of PRV labeling changed with survival time after injection (**Figure 19 & Table 2**). At day one, two cases had solely OC labeling without any labeling in the CN (**Figure 20A**). This observation suggests that PRV labeling is via the retrograde direction, rather than via auditory nerve fibers in the anterograde direction. Also in support of this view, no labeling was seen in auditory nerve fibers entering the cochlear nucleus, and labeling was not seen in spiral ganglion cells in the two cochleas that were processed (both off 2-day survival times). At later times, the numbers of OC neurons **(Figure 19**) increased and then formed a plateau. In the plateau, OC labeling averages 177.6 neurons per animal (range 8–639, SD 185.3, n=17 animals with survival times 3–5 days). MOC neurons outnumbered LOC neurons (average 122.5, SD 141.8 vs. 55.1, SD 63.5, survival times 3–5 days) and there was large variability from case to case **(Table 2).** There was no clear relationship between the injection volume and the number of OC neurons labeled or the relative proportions of labeled MOC and LOC neurons (data not shown). In contrast, HRP labeling yields an average of 1,313 neurons per animal (range 729–1997, SD 572.1, n=3 animals) even though the injection methods were similar. Both tracers labeled MOC and LOC neurons bilaterally in the brainstem, with MOC neurons primarily contralateral and LOC neurons primarily ipsilateral. For MOC neurons, 54.7 % of the PRV-labeled neurons were on the brainstem side contralateral to the injection (25 injections) and 64.7 % of the HRP-labeled neurons were contralateral. For LOC neurons, 90.2 % of the PRV-labeled neurons were ipsilateral and 94.6 % of the HRP-labeled neurons were ipsilateral.

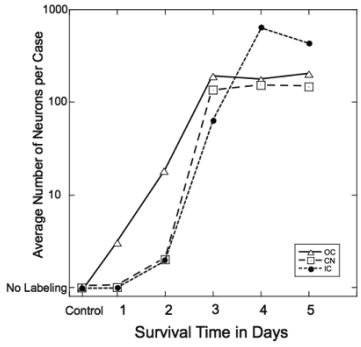

Figure 19. PRV labeling as a function of post-injection survival time
Number of labeled neurons as a function of survival time after injection (OC olivocochlear, CN cochlear nucleus, IC Inferior colliculus).

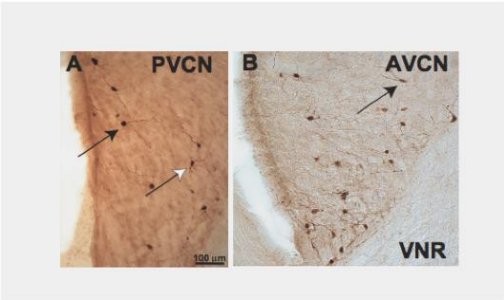

Figure 20. Photomicrographs of PRV-labeled multipolar neurons in PVCN (A) and AVCN (B) Planar multipolar cells (black arrows) have long dendrites mostly contained in a single plane and cell bodies of medium size (see text for measurements). Other multipolar cells have more radiating dendrites (white arrow). Both A and B are from the CN ipsilateral to the injection and both had a 3-day survival time. VNR vestibular nerve root. These sections are from a point about midway in the total rostro-caudal extent of the PVCN and AVCN.

9.2.4 PRV labeling of non-olivocochlear structures

Apparent retrograde labeling of a few neurons that were not olivocochlear was observed but infrequently. In three cases, one of which was a 2-day survival time, there was scattered labeling of a few vestibular efferent neurons (bilaterally, near the facial genu and/or in the pontine reticular formation, Strutz 1982). One of these PRV cases had a few labeled neurons in the ipsilateral medial vestibular nucleus, but their other patterns of labeling were not different. Our HRP-labeled cases also had retrograde labeling of vestibular efferents, and their numbers were usually greater than for PRV labeling. In four other PRV-injected cases, there were small numbers of labeled neurons in the facial motor nucleus on the ipsilateral side, presumably via spread of the virus to the facial nerve that courses adjacent to the middle ear. The cases were distributed across the survival times used. Since they did not differ from other cases in the labeling pattern seen elsewhere, they were included in our database. One case had labeling in the vicinity of the inferior salivatory nucleus (Contreras et al. 1980; Spangler et al. 1982).

9.2.5 Spread of PRV labeling to other brainstem areas at increasing survival times

PRV labeling spread to other brainstem areas for survival times of 3 days and longer. This transneuronal labeling began a day or two after the labeling in OC neurons. The most rapid transneuronal labeling occurred in the reticular formation of the brainstem, and the most sluggish was in the nuclei of the lateral lemniscus. A comparison of the number of labeled OC neurons, CN neurons, and IC neurons is shown in **Figure 19**. Labeling of OC neurons began on day 1, then increased and formed a plateau from day 3. Labeling in the CN and the IC began on day 2 and then increased to form a plateau from day 4. Thus, the labeling in CN and IC lagged behind that in MOC neurons by one day for both its initial appearance and its formation of a plateau. The maximum number of labeled CN neurons per animal was about 150, similar for MOC neurons. The

maximum number of labeled IC neurons per animal was between 400 and 700.

The labeling pattern of OC neurons varied from case to case but most had labeled MOC and LOC neurons. Within the CN, most labeling was in the AVCN subdivision with the remainder evenly divided between PVCN and DCN. Labeling in the CN was heaviest on the injected side. In the IC, the number of labeled neurons was also correlated with the number of labeled MOC neurons**.** Other studies (Faye-Lund 1986; Thompson and Thompson 1993; Vetter et al. 1993; Groff and Liberman 2003) suggest that descending projections from the IC travel to MOC neurons on the same side of the brain. The correlation between contralaterally located MOC neurons and contralaterally located IC neurons was strong but there was also a high correlation with ipsilaterally located IC neurons.

9.2.6 Transneuronal PRV labeling in various CN "multipolar" cells

The reaction product within the cochlear nucleus (CN) neurons was dark and the filling of their processes was good enough for them to be identified. The classified neurons were almost all multipolar neurons **(Figure 20)** because they have three or more dendrites protruding at widely divergent angles from their somata (Hackney et al. 1990). We classified the 1,200 neurons from the ventral cochlear nuclei on both sides in the four animals with the heaviest labeling. Of these neurons, 50.0 % were multipolar. Many others (49.1 %) had a similar appearance although they could not be definitively classified, either because their dendrites were not available in the section that contained their cell bodies or because of light labeling of their dendrites. The remaining small numbers of neurons were bushy (0.5 %) and octopus cells (0.4%) as defined by Hackney et al. (1990). Almost all labeled neurons were located in the core of the CN rather than near its edges. In particular, there was almost no labeling in the edge region known as the superficial layer of granule cells **(Figure 21)** or in the cap of small cells at the dorsal-most edge of AVCN just below this layer. In the DCN, the small amount of labeling was limited to multipolar and fusiform cells and a few giant and cartwheel cells.

The predominant subtype of transneuronally labeled multipolar cell had a medium-sized cell body and dendrites with an orientation that was flattened in an isofrequency plane (**Figure 20A & B**, black arrows). Cells showing this morphological trait were described by Doucet and Ryugo (1997, 2006) as "planar cells." The somata of these cells averaged 214.6 μm2 in area (SD 54.2 μm2, n=39 cells, average major axis 19.9 μm and average minor axis 13.0 μm). Their dendrites extended up to 150 μm from the soma. Our counts indicate that 64.6% of the neurons classified as multipolar neurons were planar cells. The remaining multipolar neurons (35.4 %) had dendrites radiating from the cell body in diverse directions (**Figure 20B**, white arrow). These somata were also medium in size (average area 205.3 μm2, SD 62.1 μm2, not significantly different from planar cells). We did not observe labeling of the largest "radiate" multipolar cells cells (Doucet and Ryugo 1997). The distribution of planar cells usually extended through both AVCN and PVCN, although some cases had most of them in PVCN **(Figure 21A)** and other cases had most of them in AVCN (**Figure 21B)**. Planar cells were usually found on both the ipsilateral and contralateral sides, but there were cases in which one side had more labeling of all types of cells.

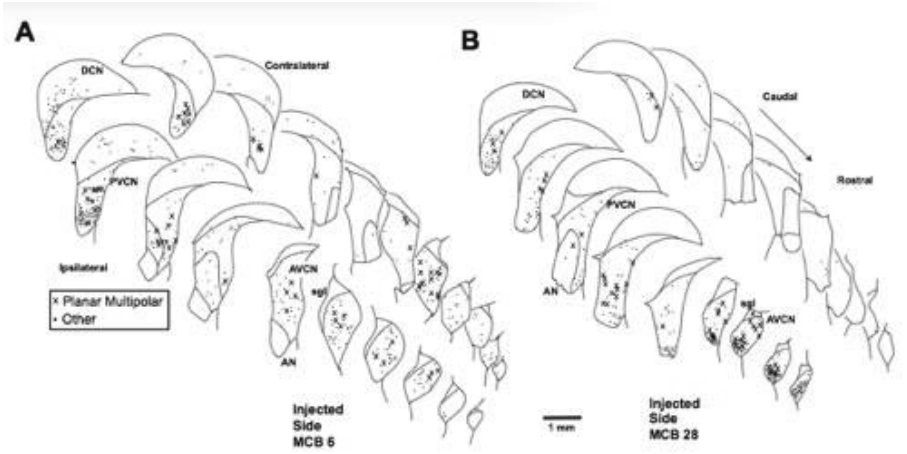

Figure 21. Atlases showing the rostro-caudal pattern of PRV labeling in CN for two injection cases
A: A case with abundant labeling in MOC neurons (n=466) and some labeling in LOC neurons (n=31). **B:** A case with abundant labeling of bothMOC neurons (n=172) and LOC neurons (n=134). Each drawing shows the superimposed labeling from four sections and each symbol represents a single labeled neuron (see key for cell type). Both the ipsilateral (injected) and contralateral sides are shown. Survival times: A, 4 days; B, 3 days. DCN dorsal cochlear nucleus, PVCN posteroventral cochlear nucleus, AVCN anteroventral cochlear nucleus, AN auditory nerve, sgl superficial layer of granule cells.

9.2.7 Transneuronal PRV labeling of disc shaped and stellate cells in the IC

In the central nucleus of the IC, where the cell types have been best-studied, PRV labeling is observed in neurons with disc-shaped dendritic fields as well as in stellate cells that have more radiating dendrites **(Figure 22).** The "disc" of the disc shaped neurons is formed of dendrites that are flattened in the dorso-medial to ventrolateral plane, the plane of the colliculus' fibro-dendritic laminae. These dendrites extend as much as 150 μm from the soma. Cell bodies of disc-shaped neurons are small/medium in size, with 133.7 μm^2 avg. area (n= 15 cells, avg. major axis 16.5 μm and avg. minor axis 11.3 μm). Labeled disc-shaped cells were the most numerous of the identified cells in the central nucleus. Disc-shaped cells are more concentrated in the caudal sections of the central nucleus, but otherwise the rostro-caudal pattern of IC labeling was fairly uniform. PRV-labeled stellate cells had radiating dendrites extending in most directions. Their cell bodies ranged in size although most are large, having 224.1 μm^2 avg. area (n= 18 cells, avg. major axis 29.0 μm and avg. minor axis 12.9 μm). The remainder of the labeling occurred in cells that could not be identified because of poor dendritic labeling or dendrites that were partially contained in other sections. Large, stellate-shaped transneuronally labeled neurons were also found in the lateral cortex of the colliculus. Stellate-shaped neurons were transneuronally labeled also in the nuclei of the lateral lemniscus.

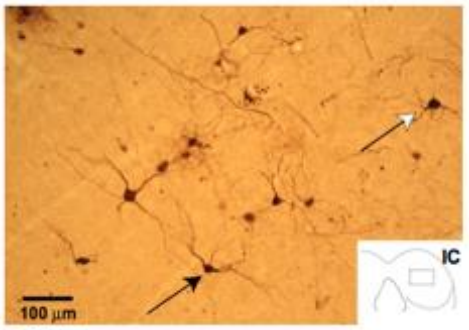

Figure 22. Photomicrograph of PRV-labeled neurons in the IC
The dendrites of the neurons indicated by the black arrows have a "disc-shaped" pattern and run along the isofrequency laminae; the neuron indicated by the white arrow has a more radiating pattern of dendrites (stellate neuron). Inset shows the position of the micrograph (in the right IC, contralateral to the injection). Survival time was 4 days. This section is from the caudal IC (one quarter of the distance from the caudal most to the rostral-most edges).

9.2.8 Transneuronal PRV labeling in the auditory cortex and other brainstem structures

Auditory cortex transneuronal labeling was observed with a time course similar to the brainstem. The labeling on the contralateral side was usually more extensive than the ipsilateral side. The labeled cortical neurons were pyramidal cells with distinct long dendrites (**Figure 23**). They were found mostly in layer V with some labeling also in layer II. One of the cases with cortical labeling had OC neuron labeling almost exclusively in MOC neurons, suggesting that cortical transneuronal labeling could take place from MOC labeling alone. Labeling in brainstem structures not typically associated with the auditory pathway was observed only for the longer survival times of 2 – 5 days and typically was most common at 3 - 5 days. Labeling was observed bilaterally in the locus subcoerulean nucleus, near the locus coeruleus **(Figure 24).** These labeled neurons were large and multipolar. The labeling sometimes extended into the locus coeruleus. Nearby neurons in the pontine dorsal raphe were also transneuronally labeled. Their medium-sized, elongated cell bodies and dendrites were vertically (dorso-ventrally) oriented along the midline of the brain. More ventrally, the pontine reticular formation had scattered bilateral labeling. Labeling here occurred in a higher percentage of cases than for the other areas and at survival times of only 2 days.

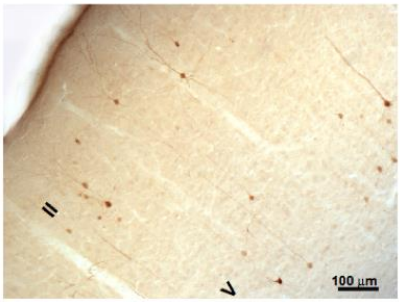

Figure 23. Photomicrographs of PRV-labeled neurons in contralateral auditory cortex
Labeling is observed mostly in pyramidal neurons of III and V after a 3-day survival time.

9.3 STUDY 3: THE THEORIES OF FABRICIUS ON MIDDLE EAR MUSCLE FUNCTION

It is important to mention a few insights from the historical study related to the works of Hieronymous Fabricius (1533-1619) who was a pioneer in approaching anatomy from a structure-function relationship. Fabricius' writings in his book "De Visione, Voce et Auditu" that I had the pleasure of translating and analyzing, provided the foundation for contemporary theories on the role of the tensor tympani because he proposed those ideas during an era when great strides were being made to increase our understanding of ear anatomy and physiology. Furthermore, it is important to consider that the basic science research into the structure and function of the auditory peripheral reflex pathways is a present day attempt to answer the same questions that occupied Fabricius' mind. In that spirit of historical foresight, I will only briefly touch on the theories he had illuminated.

9.3.1 De Visione, Voce et Auditu (1600)

Fabricius was the first scientist to hypothesize on the physiology of the tensor tympani. He presented his theories on ear anatomy and hearing in a volume of *De Visione, Voce et Auditu* (1600) called *De Aure Auditus Organo* (*Regarding the ear, the organ of hearing*). His ideas on tensor tympani function are found in Chapter 6 entitled *De musculo malleum ad incudem mouente* (Regarding the muscle that moves the hammer to anvil), in the *Pars prima* (First part) of the volume called the *De Dissectione Et Historia* (*Regarding dissection and enquiry*). Additional discussion on tensor tympani function is found in Chapter 6 of the *Pars tertia* (third part) of the volume called *De musculo et articulationis malleus utilitatibus* (Regarding the muscle and the usefulness of the hammer joint) in a section entitled *De utilitatibus* (Regarding its advantages). We had included from both chapters a number of translated excerpts from the original Latin text to help organize the number of theories postulated by Fabricius. These theories can be segregated into 1) the anatomy of the tensor tympani and the role of the tensor tympani in 2) tympanic membrane protection and 3) middle ear ventilation.

9.3.2 Fabricius and tensor tympani anatomy

Fabricius wrote that the two-dimensional movement of the malleus and the tympanic membrane was dependent on the actions of two muscles. Fabricius correctly proposed that the movement of the malleus was dependent on the contraction of the tensor tympani which he called the *"interior musculus"*.

9.3.3 Fabricius and his theories on tensor tympani and protection

The theories Fabricius proposed on the protective role of the tensor tympani contrast with our current understanding. Nevertheless, they helped to establish the first rudimentary form of the intensity-control theory of a middle ear muscle, which is now

attributed to the stapedius muscle. Fabricius believed that the anatomic presence of the tensor tympani shielded the tympanic membrane that was in danger of being ruptured or weakened. He described the arrangement of the muscles as forming a barrier on one side of the tympanic membrane, protecting it against both external factors. In agreement with our current understanding of tensor tympani function in humans, Fabricius did not think that the tensor tympani had a direct role in the perception of sound. He felt that the voluntary contraction of a middle ear muscle during noise attenuated the transmission of acoustic energy, and he correctly surmised that the reduction of acoustic energy affected sound perception to some degree. However, Fabricius incorrectly theorized that the tensor tympani, and not the stapedius, played the primary role in this process.

9.3.4 Fabricius and his theories on tensor tympani and middle ear ventilation

Fabricius was the first scientist to propose a non-auditory role of the tensor tympani. This is a concept that has evolved greatly since the second half of the 20th century stemming from improvements in histological techniques. Fabricius explained that in certain situations, where there was an accumulation of air in the middle ear, the tensor tympani contracted to clear the middle ear of “spoiled air”. During situations such as defecation and fever, he claimed that there was an accumulation of poor quality air in the middle ear cavity, and that tensor tympani activity helped to remove this.

10. GENERAL DISCUSSION

10.1 SPECIFIC TOPICS I

10.1.1 Distribution and number of TTMNs

In our studies of the auditory periphery (namely the middle ear muscle reflex) using chemical labeling techniques instead of a viral tracer, we discovered that the distribution of labeled TTMNs in mice was similar to that of other species such as rat and guinea pig (Lyon, 1978; Strominger et al., 1981; Spangler et al., 1982; Rouiller et al., 1986). They were observed ventro-laterally to the ipsilateral trigeminal motor nucleus as a concentrated, narrow and well defined curvi-linear column that was geographically distinct from the trigeminal motor nucleus. The column extended along the rostro-caudal axis of the trigeminal motor nucleus. These findings agree are in line with previous experiments showing that labeled TTMNs are found just outside the trigeminal motor nucleus (Lyon, 1975; Keller et al.,1983; Shaw and Baker, 1983; Spangler et al., 1982). Previous studies have referred to this pool of labeled TTMNs as being distinct from the trigeminal motor nucleus in terms of cell body size, location and function (Friauf and Baker, 1985).

10.1.2 Cell group K

The area of labeled TTMNs has been previously termed as being a separate "tensor tympani motor nucleus of V" rather than just an extension of the trigeminal motor nucleus (Hutson et al., 1979, Friauf and Baker, 1985). The location of TTMNs are thought to correlate to "Cell Group K," a specific area in the brainstem containing motoneurons that supply the masseter, digastric, and Eustachian tube muscles (Donga, Dubuc, Kolta, & Lund, 1992; Reuss, Kuhn, Windoffer, & Riemann, 2009; Saad, Dubuc, Westberg & Lund, 1999). Interestingly, it has been demonstrated in cat that only 700 TTMNs supply approximately 4,000 tensor tympani muscle fibers (Blevins, 1964; Shaw & Baker, 1983) to give the tensor tympani an innervation ratio of 1:5.7. Unlike SMNs, TTMNs have not been shown to be spatially located around the trigeminal motor nuclei according to their individual physiological responses to stimuli.

10.1.3 Different types of TTMNs

Based on morphological characteristics, labeled mice TTMNs were identified and separated into three distinct subtypes, which we called "octopus-like", "fusiform", and "stellate" TTMNs. A few intermediates between "octopus-like" and "fusiform" subtypes may exist. "Octopus-like" TTMNs were the most common and "stellate" TTMNs were the least common. These different subtypes may reflect differences in TTMN function similar to that seen in the cochlear nucleus, an important brainstem structure essential for

central processing of auditory impulses (Kiang et al., 1973; Rhode et al., 1983; Rouiller and Ryugo, 1984; Adams, 1986). Different shapes and morphologies might account for the multiple functions of TTMNs in response to auditory and non-auditory stimuli. Alternatively, the different subtypes might innervate different types of muscle fibers, such as the slow, fast and medium twitch muscle fibers.

10.1.4 Dendritic characteristics of motoneurons

The TTMN dendrites were observed to be long and sparsely branched. The study of dendrites is important because the orientation and distribution of dendritic spread have been shown to influence neuronal response properties (Sotnikov, 2005; Berkowitz et al., 2006; Hickmott and Ethell, 2006; Saxon and Hopkins, 2006; Torres-Fernandez et al., 2007; Bergquist and Ludwig, 2008). Furthermore, proximal dendrites of TTMNs receive abundant synaptic input, but there is sparse innervation of the TTMN cell body (Lee et al., 2009). Distal dendrite synapses have not been investigated, but since the distal dendrites project extensively (Friauf and Baker, 1985), they present a large surface area on which to receive inputs. These inputs come from the cochlear nucleus (Billig et al., 2007), from serotoninergic sources (Thompson et al., 1998), and presumably from motor control areas. This probably reflected in the diversity of tensor tympani muscle activity in response to both auditory and non-auditory stimuli. The prominent dendritic labeling that was achieved using FG allowed identification of specific characteristics of TTMN dendrites in mice. Mouse TTMN dendrites extended for long distances beyond the region of labeled cell bodies. This was similar to TTMN dendrites in cats (Friauf and Baker, 1985). In both species, TTMN dendrites avoided crossing into the trigeminal motor nucleus (Friauf and Baker, 1985). Possibly the distinct organization of TTMN dendrites reflects underlying physiological differences between the pool of TTMNs and the motoneurons of the trigeminal motor nucleus (Friauf and Baker, 1985). Our study is the first to systematically quantify the TTMN dendrites and also to measure their lengths and angle of orientation. The dendrites extended in a radial pattern in all directions from the column of labeled TTMNs. Dendrites directed dorso-medially were more numerous and were the longest in length. A similar numerical and length bias is present for dendrites of medial olivocochlear (MOC) neurons (Brown and Levine, 2008). MOC neurons receive dominant inputs from the opposite side onto their medially projecting dendrites; the similar numerical and length bias suggest that this is true also for TTMNs.

10.1.5 Synaptic innervation of motoneurons

Previous work from our laboratory, has determined the types and richness of the synaptic inputs that fall onto the distal dendrites of retrogradely labeled rat TTMNs using electron microscopy (D. J. Lee et al., 2008). The frequency and morphology of terminal types observed on TTMNs were similar to that seen on SMNs (Benson et al., 2008; D. J. Lee et al., 2008). Synaptic terminals on TTMNs were classified into the four terminal types according to the size and shape of the synaptic vesicles. They were named (a) large round, (b) small round, (c) pleomorphic, and (d) heterogeneous. Compared with SMNs, there were slightly fewer small round terminal types found on TTMNs (D. J. Lee et al., 2008; Benson et al., 2013). SMNs also received a rare Cistern-type terminal that was not

seen on TTMNs (D. J. Lee et al., 2008). In TTMNs, terminal types with round vesicles (large round and small round) constituted 40% to 45%; Conradi, Kellerth, Berthold, & Hammarberg, 1979). The relatively high proportion of terminal types containing round vesicles signifies that the TTMNs receive mainly excitatory input (Uchizona, 1965). The presence of some pleomorphic terminal types suggests that TTMNs also receive inhibitory input. As in the case of SMNs, the assignment of TTMN terminal types to the cell bodies of origin has not been established. The CN, SOC, serotoninergic sources, and higher cortex are all possible sources (Billig et al., 2007; Gelfand, 1984; Stach et al., 1984; Thompson
et al., 1998).

10.2 SPECIFIC TOPICS II

10.2.1 Time course of PRV labeling of OC neurons

The time course of PRV labeling is consistent with a direct labeling of OC neurons because staining occurred after short survival times. The transneuronal labeling of other neurons occurred after longer survival times only, and is therefore consistent with transport of labeling via synaptic inputs. In our study and that of Horvath et al. (2003), PRV labeling first appears in OC neurons at 1-2 days after viral administration, and with another day of survival then spreads to other neurons, such as those in the auditory centers such as the CN and IC. It also spreads to neurons in non-auditory centers such as the reticular formation and locus subcoeruleus. This time course of labeling is consistent with the documented retrograde direction described for PRV transport. Also consistent with this finding is the absence of labeled auditory nerve fibers in CN and no labeled spiral ganglion cells for survival times after 2 days, when there was abundant labeling of OC neurons. Horvath et al. (2003) describe similar results though they reported a few labeled spiral ganglion cells for longer survival times.

10.2.2 Quality and quantity of PRC labeling of OC neurons

Our study indicates PRV labeling of only about 15% of OC neurons. Similar results were found by (Horvath et al. 2003). They reported PRV labeling of 560 - 724 LOC neurons (the MOC number was not given), which is less than the numbers of OC neurons labeled by traditional tracers in the guinea pig (Strutz and Bielenberg 1984; Aschoff and Ostwald 1987). Like the present work, Horvath's work found significant variability in the numbers of labeled OC neurons and a significant number of injections that failed to label any OC neurons. There are some differences in our present results and those of (Horvath et al. 2003). In our study, labeling appeared with about one day longer time course. The reason for this difference is unknown. Also, they report a 2:1 contra:ipsi ratio of MOC neurons, whereas our ratio was closer to 55%:45%. Horvath reported on MOC neurons that were only located in VNTB, even though HRP-labeled MOC neurons are present also in DMPO.

10.2.3 Identity of cochlear nucleus cell types providing inputs to OC neurons

In the CN, multipolar cells are the neurons that are transneuronally labeled (Horvath et al. 2003s). Our results suggest that this transneuronal labeling proceeds via MOC neurons, since there is a correlation between their numbers and the number of labeled CN neurons. A major subtype of transneuronally labeled neurons are the planar cells. This subtype projects to MOC neurons, as shown by anterograde tracing of dextran-amine-labeled axons (Darrow et al. 2012). Present results indicate a high correlation (R=0.91) of MOC labeling and PVCN transneuronal labeling. Neurons of the PVCN project to MOC neurons (Thompson and Thompson 1991), and lesions there interrupt the MOC reflex (de Venecia et al. 2005). Planar cells have a distribution that includes PVCN (Doucet and Ryugo (1997). Thus, available data point to the conclusion that planar cells of PVCN function as the intermediate neurons of the MOC reflex. This idea fits with the fact that planar cells have dendritic fields that are narrow in the isofrequency plane of the CN (Doucet and Ryugo (1997). Their physiological correlate, "chopper units", like MOC neurons, are almost as sharply tuned to sound frequency as auditory nerve fibers (Godfrey et al., 1975; Robertson 1984; Liberman and Brown, 1986). A new finding from present work was the large amount of labeling in AVCN that appeared at the same time as the PVCN labeling. There was a high correlation (R=0.86) of MOC labeling and AVCN transneuronal labeling. There are these inputs from AVCN but they are maybe weaker and not able to drive the MOC response to sound, as has been shown for unidentified facilitatory inputs in physiological experiments (Liberman 1988). Another possible source for some of this AVCN labeling could be LOC neurons. However this did not show up in our correlations.

It was also surprising that not much transneuronal labeling was observed in the superficial layer of granule cells or in the cap of small cells just below this layer. Together, these edge regions in AVCN are known as the "shell", and in the cat its neurons project to OC neurons (Ye et al. 2000). The reason for the lack of transneuronal labeling in our studies is not clear but could be due to species difference.

10.2.4 Ipsilateral dominance of cochlear nucleus cell neuron labeling

One difference between the present results and those of Horvath et al. (2003) is that they found a slight contralateral dominance for CN labeling, whereas we found a large ipsilateral dominance (78.5%). Our results fit with the finding that the MOC reflex driven by ipsilateral sound is stronger than that driven by contralateral sound, and that the former proceeds via neurons in the ipsilateral CN that project to MOC neurons on the opposite side of the brainstem (Liberman and Brown 1986; Liberman and Guinan, 1998). We found, though, that the correlation of MOC neurons on one side with the CN labeling on the other side was not particularly high. Perhaps this reflects the fact that nondominant- ear, facilitatory inputs are numerous (Liberman 1988; Brown et al. 1998).

10.2.5 Transneuronally labeled cells in the middle ear muscle reflex

Middle ear muscle injections of PRV yield different types of transneuronally labeled neurons. For tensor tympani muscle injections, there is labeling of large neurons

with long, radiating dendrites (although longer survival times yield a wider variety of neurons Billig et al. 2007). Present results do not label these radiate multipolar cells, some of which have been reported to project to the opposite CN (Doucet and Ryugo 2006; Brown et al, 2012). Stapedius muscle injections of PRV yield a mixture of cell types in CN, and labeling is seen at earlier times in the superior olivary complex (Windsor et al. 2007). Our cochlear injections did not produce the "cytopathic" changes reported by (Billig et al. 2007), which were invaginated nuclei, neurons containing inclusion bodies, somata with irregular shape, or beaded dendrites, but perhaps our observed "clouds" of reaction product (Fig. 1B) signify the end-stage of this process.

10.2.6 PRV labeled inputs from the inferior colliculus

Our work demonstrated transneuronal labeling in both major types of collicular neurons, stellate neurons and disc-shaped neurons, which thus provide descending projections to OC neurons. Both of these types form ascending projections to the medial geniculate body (rev. by Oliver and Huerta 1992). However, we do not know whether the particular cells that form ascending connections also form the descending projections labeled in the present study. Descending inputs from IC to OC neurons were demonstrated previously by transneuronal labeling (Horvath et al. 2003), with conventional tracers (Faye-Lund 1986; Vetter et al. 1993), and by electric stimulation (Groff and Libeman 2003; Ota et al. 2004), but the cell type of this projection had not been previously known. The correlation between the number of MOC neurons and the number of transneuronally labeled IC neurons suggests that transneuronal labeling proceeds via MOC neurons. One difference between our study and the work of Horvath et al. (2003) is that they found labeling confined to the central nucleus while we found a limited amount of labeling in the dorsal and lateral cortices. Like Horvath et al. (2003), we found that the IC labeling had a slight contralateral dominance.

10.2.7 PRV labeled inputs from cortex and other centers

In the auditory cortex, transneuronal labeling appears in pyramidal cells after cochlear injections of PRV (present results and those of Horvath et al. 2003). Labeling was present in cortical layer 5, similar to Horvath et al. (2003), and we also found some labeling in layer 2. These layers are the origin of most descending projections from cortex to lower centers (rev. by Winer 1992). Descending inputs from layer V of cortex to the MOC neurons have been described (Mulders and Robertson 2000a). For our limited cortical material, we cannot rule out labeling that proceeds via an indirect pathway involving another center such as the inferior colliculus. The indirect pathway is suggested by the fact that Horvath et al. (2003) saw cortical labeling only at the longer survival times. Labeling in medial geniculate has seen previously (Horvath et al. 2003) and to a limited extent in the present study.

10.2.8 Transneuronal labeling of "nonauditory centers"

Transneuronal labeling seen in our study indicates involvement of the "nonauditory" centers, the pontine dorsal raphe, the reticular formation, and the subcoerulean nucleus in the olivocochlear pathway. One difference between present results and those of Horvath et al. (2003) is that they observed reticular formation labeling ("scattered and specifically labeled cells in the pontine reticular formation") at very short times after injection (1 day) It was present in a few cases even without OC neuron labeling. In our study, this labeling never appeared without OC labeling, and, although it was the earliest non-OC center to label, we did not find it until 2 days. We cannot explain this difference, although Horvath et al. (2003) generally used larger injection volumes (50 ul), which may have spread to non-cochlear nerve endings. Also, perhaps some vestibular efferent neurons scattered in the dorsal pontine reticular formation (Strutz 1982), which we occasionally noticed, may have previously been counted as general reticular labeling. Immunohistochemical studies show that noradrenaline-containing varicosities are in contact with both MOC and LOC neurons (Woods and Azeredo 1999; Mulders and Robertson, 2000b). The source of this noradrenergic innervation is the locus coeruleus in the rat (Mulders and Robertson 2001) and the subcoerulean nucleus in the guinea pig (Mulders and Robertson 2005). These non-auditory inputs represent important pathways to OC neurons, but their functional role in the reflex pathways and in hearing remains to be clarified.

10.3 SPECIFIC TOPICS III

10.3.1 Theories of protection 350 years later

Fabricius was correct in ascribing a protective function to a middle ear muscle. However, at the turn of the 20th century, a new method of measuring middle ear function challenged the idea that the TT was the dominant middle ear muscle in man. In the 19th century, an English scientist named Oliver Heaviside (1850-1925) helped to develop the concept of acoustic impedance for auditory research (Nahin et al., 1987). Through his work on telecommunications, Heaviside introduced the role of resistance or impedance within the series circuitry of telephones in 1886 (Heaviside, 1894). It was his application of a mathematical formula that helped define impedance in objective terms (Heaviside, 1894). The first device for measuring the acoustic impedance in patients was conceived in Scandinavia in 1939 by the German-born Danish scientist Otto Metz (1905-1993) (Metz, 1952). Otto Metz developed his device on the basis of a paper published by E. Waetzmann in 1938 that introduced a modified version of an impedance measuring appliance. This measuring device was invented in 1934 by K. Schuster and was originally conceived to study the absorption of sound in construction material. Metz's own device, called The Metz Acoustic Bridge, detected a sound evoked pressure change in the external auditory canal (Metz, 1946). This pressure change results from the stiffening of the ossicles subsequent to middle ear muscle contraction. He was the first to use acoustic impedance as a measurement of middle ear function for diagnostic purposes in clinical otolaryngology. Metz's main interest was to distinguish conductive from sensorineural hearing loss. With the acoustic impedance meter, it was now possible to objectively

measure middle ear function in patients with different ear pathologies. Later studies using the acoustic impedance meter would demonstrate that the stapedius and not the tensor tympani was the dominant acoustically evoked middle ear muscle in man (Jepsen et al., 1955). For example, patients with transected stapedial tendons showed no measurable acoustic impedance (Brask, 1979). However, the acoustic impedance was observed in ears of patients with transected tensor tympani tendons. Furthermore, in patients with Bell palsy (and a paralyzed stapedius muscle), there was no evoked middle ear muscle activity when the affected ear was exposed to sound. However, impedance changes were measurable in patients with a non-functioning tensor tympani secondary to trigeminal nerve paralysis. Although Fabricius overlooked the protective role of the stapedius muscle, he helped establish the foundations for future middle ear muscle research.

10.3.2 Theories of middle ear ventilation 350 years later

The theory proposed by Fabricius that the tensor tympani plays a possible role in middle ear ventilation remains relevant today. The tensor tympani and other Eustachian tube muscles such as the dilator tubae and tensor veli palatini have been shown to be histologically continuous. The close anatomic relationship amongst these muscles suggests that they are components of the same functional unit involved in the opening and closing of the eustachian tube (Rood et al., 1978). A recent Austrian study has also demonstrated the presence of collagenous connections between the tensor tympani and tensor veli palatini (Kierner et al., 2002). They are known to be embryologically similar and receive the same motor innervation (Rood et al., 1978). Fabricius' theory on the nonauditory role of the tensor tympani to maintain middle ear barometric pressure is perhaps his most important contribution to our understanding of the function of the tensor tympani in humans.

10.4 SPECIFIC TOPICS IV (UNPUBLISHED RESULTS)

10.4.1 Importance of transneuronal analysis in ABI development

The importance of conducting a quantitative and qualitative analysis of the auditory reflex pathways in the brainstem touches on a clinical relevance. The findings of the PRV labeling study of the OC reflex pathway postulated that the majority of the CN interneurons providing inputs to the MOC and LOC neurons exist in the PVCN (and the AVCN). The labeled CN neurons have been identified as being "radiate multipolar" of the "planar" type since their dendritic orientation appears to stretch along single iso-frequency planes within the CN. Labeling studies that successfully identify the various afferent and efferent components of the auditory reflex pathways will help to improve future auditory prosthetic devices as discussed in section 6.6.4. Transneuronal analysis offers a better understanding of the neural circuitry within the auditory brainstem to improve surgical placement and impulse processing of auditory brainstem implants (ABI). Since an ABI bypasses a damaged auditory nerve (such as in NF-2 patients) and directly stimulates the CN, increased knowledge of the CN and its neural interaction with other reflex stations in the brainstem becomes more valuable.

10.4.2 Current ABI technology

ABI technology utilizes electrodes that deliver electrical current to the neurons of the CN. A large majority of ABI users obtain an awareness of sound that enhances meaningful communication when assisted by lip reading. However many of these patients struggle to understand meaningful open-set words (Otto et al., 2002). In addition, some of these patients experience uncomfortable non-auditory sensations such as taste disturbances or facial pains from the ABI due to non-specific stimulation of neurons that are located near the CN (Otto et al., 2002; Shannon et al.,1993; Shannon et al.,1997).

10.4.3 Infrared (optical) neural stimulation

In addition to the transneuronal labeling studies, our laboratory examined alternative methods for stimulating the CN in an effort to find ways to improve the experience of ABI users (Mukerji & Lee., 2009) This was achieved by the use of low amplitude power mid-wavelength infrared neural stimulation (INS) off CN neurons (Izzo et al., 2006:Wells et al., 2007). INS (or optical stimulation) is dependent on a laser that transports energy through an optic fiber. INS has been successful previously in directly stimulating the spiral ganglion cells in the cochlea of gerbils (Izzo et al., 2006). In these gerbil experiments, optically evoked compound action potentials were observed during six hours of continuous exposure with INS to the cochlea. Interestingly, unlike electrical stimulation that can cause current spread across a large number of neurons (Frijns et al., 1996), INS appeared to only activate neurons that are located within the path of radiant energy. The exact mechanisms responsible for neural activation following laser stimulation are as yet unknown. One proposed theory is that the transient temperature rise

from laser exposure creates an influx of ions and the consequent depolarization of neurons (Izzo et al., 2007).

10.4.4 Optical stimulation of the auditory brainstem: unpublished results

The aim of our INS experiments was to determine the feasibility of using INS to activate neurons in the CN in an acute animal model such as the rat (Mukerji & Lee., 2009). This was achieved in part based on the knowledge of the auditory reflex pathways obtained through the neuronal labeling studies. The experiments formed the basis of a parametric study that explored the relationship of INS evoked auditory brainstem responses with stimulus parameters being frequency, pulse width and wavelength. Another aim was to explore the stability of the responses to long-term stimulation and also to compare electrically evoked to optically evoked auditory brainstem responses. In these experiments, the brainstem of a total of seven anesthesized rats was exposed via subocciptial craniotomies with cerebellar aspiration to expose the CN (**Figure 25)**. An optical fiber transmitting INS was placed carefully on different areas of the exposed CN in a sound proof chamber. The location of fiber placement on the CN affected the amplitude of the auditory brainstem responses. Optically evoked auditory brainstem responses were obtained from seven animals.

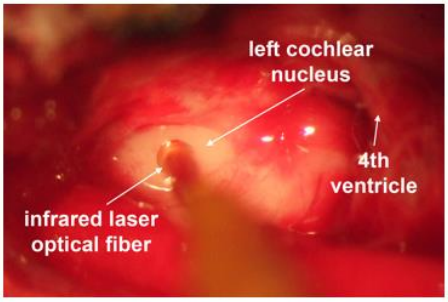

Figure 25. Photograph showing exposure of the rat brainstem following suboccipital craniotomy and cerebellar aspiration. An optical fiber contacts the lateral surface of the dorsal cochlear nucleus. Preliminary experiments determined that the responses were much smaller when the optical fiber did not contact the brainstem

Control experiments taken with the laser output blocked and after the death of the animal demonstrated that the response was indeed produced by the laser source and not by artifacts (**Figure 26)**. The strongest responses were measured when the fiber was placed at lateral positions on the PVCN/AVCN closest to the temporal bone.

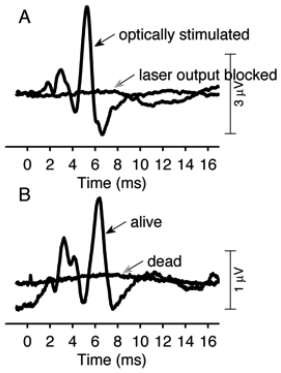

Figure 25. Photograph showing exposure of the rat brainstem following suboccipital craniotomy and cerebellar aspiration. An optical fiber contacts the lateral surface of the dorsal cochlear nucleus. Preliminary experiments determined that the responses were much smaller when the optical fiber did not contact the brainstem

There was a clear parametric dependence of the evoked response in which the amplitude of the response increased with increasing radiant energy and decreasing wavelength (**Figures 27 & 28**). There was little or no relationship between the response and the pulse width of the laser. The response was stable over a protracted period of time with continuous pulsed stimulation. Post experiment histology of the CN following longterm optical stimulation showed no cell loss or necrosis in the regions of the CN that were stimulated. A later study by the same laboratory group published results that also showed that pulsed INS delivered to the surface of the CN via a similar optical fiber evoked broad neural activation as far up as in the inferior colliculus in a rat model (Verma et al., 2014).

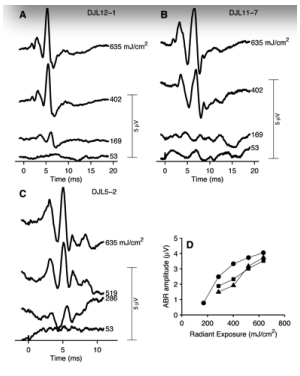

Figure 27. oABR waveforms at different radiant exposures. Waveforms A, B and C (taken from 3 different animals) demonstrate that oABR amplitudes increased with increasing radiant energy. As exposure increases, the largest peak (p2) increases in amplitude and other peaks emerge. D) amplitude of the largest oABR peak (p2) as a function of increasing radiant exposure. Amplitude increases with exposure, approximately linearly.

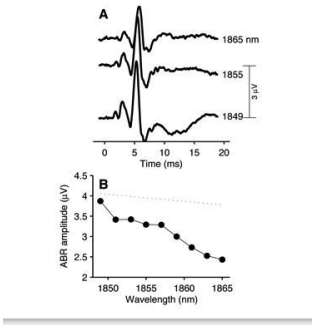

Figure 28. oABR waveforms at different stimulus wavelengths. A: Example of waveforms taken from one animal demonstrate that oABR amplitudes increased with decreasing wavelengths. Amplitude is largest for the shortest wavelength. **B:** Graph showing OABR peak (p2) as a function of increasing stimulus wavelength. Amplitude of the measured oABR decreases with increasing wavelength. This can be explained by the fact that energy disperses from the surface with increasing wavelength.

10.4.5 Optical stimulation of the auditory brainstem: unpublished conclusions

The results from the INS experiments, though not published as yet, highlight the importance of locating and analyzing the morphometric and physiological characteristics of CN interneurons. Transneuronal analysis of the auditory reflex pathways assists in the identification of crucial neurons within the OC and MEM reflex pathway that can assist in the evolution of ABI. Locating the CN , OC or MEM reflex interneurons will also assist in better placement of electrical electrodes (or optical fibers if INS becomes a feasible component in future ABI design). By placing electrodes onto areas that elicit the

strongest auditory brainstem response, the efficiency of ABI and ultimately hearing outcomes of potential patients can be greatly improved. It has been shown already that most of the inputs that synapse onto MOC neurons arrive from the PVCN/AVCN neurons. Therefore optimal placement of electrodes to stimulate only this collection of neurons can theoretically improve the patient's ability to locate acoustic signals and to also minimize unwanted non-auditory sensations. Additionally, assessing the middle ear muscles during actual ABI placement will provide the clinician with a potential external measure through which the integrity of the auditory reflex pathways can be gauged in ABI users.

11. MAIN CONCLUSIONS

- The tensor tympani is one of two middle ear muscles that regulates the transmission of sound through the middle ear. The tensor tympani and the stapedius are known to contract in response to acoustic stimulation in most mammals. In humans however, the stapedius is the primary acoustically driven middle ear muscle. Various non-acoustic theories on tensor tympani function have been formulated since the 16th Century that allude to its role in maintaining ventilation off the middle ear cavity. Contraction of the tensor tympani in response to both auditory and non-auditory stimuli is mediated by the efferent component of the tensor tympani reflex. The efferent (or motor) component of the reflex consists of tensor tympani motoneurons (TTMNs). There are interesting differences among animal species in the acoustic thresholds for contraction of the middle ear muscles, which may be a reflection of underlying anatomical differences such as the number, orientation and location of TTMNs. Our study investigated the number and morphology of TTMNs in mice (a common animal used in hearing research) using Fluorogold, a retrograde trans-synaptic neuronal tracer. After injections of Fluorogold into the tensor tympani muscle, a column of labeled TTMNs was identified ventro-lateral to the ipsilateral trigeminal nucleus. The location of labeled TTMNs in the mice model using Fluorogold was similar to other labeling studies using alternative labeling techniques such as HRP (horse radish peroxidase). Labeled TTMNs were theorized to occupy an area distinct from the trigeminal motor nucleus called “separate nucleus of the tensor tympani”. Labeled TTMNs were classified according to their morphological characteristics into three subtypes: “octopus-like”, “fusiform” and “stellate”. Distinct morphological subtypes were theorized to reflect underlying differences in neuro-physiological function such as in cochlear nucleus neurons. The variance in subtypes and consequent function can explain the diversity of tensor tympani response to both acoustic and non acoustic stimulation. In 18 cases, the mean number of mouse TTMNs was 51; the largest numbers were 70, 74 and 90. The mean size of mouse TTMNs was 13.0 μm (minor axis) and 23.5 μm (major axis). Compared with studies of TTMNs in larger species (cats and rats), mouse TTMNs are both fewer in number and smaller in size. All three subtypes formed far reaching sparsely branched and radiating dendrites. Some of these dendrites were far reaching and extended longer than 600 μm across the brainstem, avoiding the borders of the trigeminal motor nucleus. Dendrites were longest and most numerous in the dorso-medial direction. The longest dendrites extended into regions of the brainstem, such as serotonin rich regions, that were not predominantly associated with processing of auditory regions. Thus suggesting that tensor tympani motoneurons obtain impulses from non-auditory centers in the brainstem.

- Olivocochlear (OC) neurons of the superior olivary complex (SOC) respond to sound and provide descending input that controls auditory processing in the cochlear nucleus. They assist our abilities to localize acoustic sources through the neuronal mediated analysis of inter-aural amplitude and time differences of sound. The retrograde transneuronal tracer pseudorabies virus (Bartha strain: expressing green fluorescent protein) has been proven to be an effective trans-synaptic labeling method. PRV was used to label both medial and lateral OC neurons and their inputs across the various brainstem stations in guinea pigs. MOC neurons were the pre-dominant and largest of the labeled neuron of the SOC and were found mainly in the ventral nucleus of the trapezoid body and dorso-medial periolivary nucleus. Labeling of OC neurons began one day after injection into the cochlea. On day 2 (and for longer survival times), transneuronal labeling spread progressively to the cochlear nucleus, inferior colliculus, and other brainstem areas such as the cortex. There was a correlation between the numbers of transneuronally labeled neurons and the number of labeled medial (M) OC neurons, suggesting that the spread of labeling proceeds mainly via synapses on MOC neurons. In the cochlear nucleus, the transneuronally labeled neurons were multipolar cells including the subtype known as planar cells. These planar cells were orientated across single iso-frequency planes within the cochlear nucleus. The most labeled cochlear nucleus neurons were found in the AVCN although cells were also seen in the PVCN and DCN. Labeling spread to other brainstem areas for survival times of 3 days and longer. This transneuronal labeling began a day or two after the labeling in OC neurons. Transneuronal labeling was observed in the inferior colliculus, the pyramidal cells in the auditory cortex and in centers not typically associated with the auditory pathway such as the pontine reticular formation, subcoerulean nucleus, and the pontine dorsal raphe. The data provides information on the identity of neurons providing input to OC neurons, which are located in auditory as well as non-auditory centers. The most rapid transneuronal labeling occurred in the reticular formation of the brainstem, and the most sluggish was in the nuclei of the lateral lemniscus. In the central nucleus of the inferior colliculus, transneuronally labeled neurons were of two principal types: neurons with disc-shaped dendritic fields and neurons with dendrites in a stellate pattern.

- Hieronymous Fabricius (1533-1619) was a highly acclaimed academic scientist and physician. He refined methods of conducting research by relating all his observations of an anatomic structure to its function in the human body. Often referred to as the father of modern embryology for his work on understanding human fetal formation, he was also the first to publicly demonstrate the venous valves. Fabricius has also contributed greatly to our understanding of tensor tympani function in his seminal work entitled *De Visione, Voce et Auditu* published in 1600. Fabricius' published observations were the first to attribute a protective function to a middle ear muscle. He correctly observed (in himself) that the tensor tympani could be voluntarily contracted. Fabricius also suggested that the contraction of a middle ear muscle during an acoustic stimulus resulted in the diminishing of auditory perception. Most importantly, Fabricius

was the first anatomist to dedicate a non-auditory role to the TT by suggesting that the muscle played an important role in maintaining middle ear ventilation. These latter theories have provided the foundation for further study of middle ear muscle physiology. We also now know that some of Fabricius' theories on middle ear muscle function were not correct. His greatest error was in assigning the dominant protective role to the tensor tympani instead of the stapedius. He also wrote about the existence of an unknown exterior muscle and admitted that this structure was not routinely found on subsequent dissections. The precise nature of this structure is unclear. Nevertheless, on the basis of his systematic approach to studying the middle ear anatomy, Fabricius has provided the foundation for continued research on the role of the TT, the precise function of which continues to elude scientists.

- Labeling studies to delineate the afferent and efferent components of the auditory brainstem circuitry has clinical uses. Clinical applications include distinguishing between cochlear and retrocochlear pathologies in addition to assisting in the electronic processing of cochlear implants. Understanding the synaptic chains of the middle ear reflex pathway will provide the foundations on which neuro-physiological disorders such as middle ear myoclonus syndromes can be better managed. Furthermore, treatment in the form of centrally acting mediations and/or surgical interventions can be tailored according to the level at which the dysfunction is postulated to reside. Auditory brainstem implantation is an important means of providing acoustic sensation to patients with a non-functioning auditory nerve either secondary to tumor invasion or after surgery. Electronic stimulation devices used in current brainstem technology has been very successful but can result in uncomfortable side effects due to spread of activation across other brainstem areas. Infra red light (or optical stimulation) is under experimental evaluation to deem its feasibility as another means of provide frequency dependent stimulus across the cochlea nucleus. Unpublished results from the successful optical stimulation experiments using infrared light on rat cochlear nuclei demonstrate a safe and real response with parametric dependence on stimulus frequency and wavelength. Analysis of the neuronal chains within the middle ear muscle and olivocochlear reflex pathways parallel to improvements in brainstem technology will allow improvements in placement of the electrodes intra-operatively to enhance sound awareness and word recognition.

12. FUTURE PROSPECTS

A clinician working to better understand the auditory brainstem in mammals will consider the anatomy and physiology of the auditory peripheral reflexes with great interest. The objective measurement of the middle ear muscle reflex for example can potentially provide a useful tool in the accurate guidance and/or placement of auditory prostheses devices such as the auditory brainstem implant. As with many branches of neuroscience, the investigation of these complicated neural pathways relies heavily on fast evolving tracer and labeling methods such as that provided by the Fluoro-gold and the Pseudo-rabies virus. Due to the great complexity of the neuro-anatomical and neuro-physiological components of the auditory reflex pathways, both clinical and basic science research based investigations and applications should be closely integrated. This will allow both parties to accurately refine the application of each discovery and expand our knowledge of a fascinating and complex system. Through the description of published and unpublished results from the above mentioned studies, more data is made available to the research community, and thus providing the background for further exploration of the auditory reflex pathways that govern our ability to react to external aural stimulation. Although the components of the auditory periphery reflexes have been studied in great detail in the past, the ability to maintain consistent uncorrupted and uninterrupted labeling of each neural chain in the reflex is difficult. Using the recent advances in viral and chemical tracers, our studies show that it is possible (with time and patience) to satisfactorily elucidate the essential parts of the middle ear and olivocochlear reflex pathways in mammals. Further research is required to correctly identify the multitude of inter-neurons that potentially exist both within the cochlear nucleus, other areas of the brainstem and between the higher order neurons within the auditory cortex. Assessment and improvement of the replication patterns of the viral tracers, prevention of the cytopathic elements of the virus and enhancement of the longevity of chemical tracers should shed light on these problems. In the case of the tensor tympani muscle, we have successfully demonstrated that the middle ear muscles have been a source of academic enquiry since antiquity. We know from several studies, including ours, that there are still unknown interneurons in the ventral cochlear nucleus that project either directly or indirectly to middle ear muscle motoneurons located elsewhere in the brainstem. These are the motoneurons that provide efferent innervation to the middle ear muscles. Although the ascending and descending limbs of these reflex pathways have been well characterized, the precise identity of the reflex interneurons is still not known. The source of modulatory inputs to these pathways is also an unknown fundamental question. We have demonstrated that there exists a diversity of cells that control tensor tympani muscle function and also presumably interact with other non-auditory areas of the brainstem. If this is the case in the human tensor tympani reflex pathway remains to be studied. It would be of interest to further analyze the neural make up of the tensor tympani muscle to look for possible additional characteristics to help understand its auditory and non auditory functions.

The use of pseudo rabies virus as a retrograde trans-synaptic viral tracer, enabling labeling of the coherent stations of the olivocochlear reflex pathway has been explored in

many studies including ours. OC neurons respond to sound and provide descending input that controls processing in the cochlea. The precise identities of all the neurons in the pathways that provide inputs to OC neurons are still incompletely understood. We discovered labeling of OC neurons that began from the first day after injection into the cochlea and further labeling at longer survival times. There was a correlation between the numbers of these transneuronally labeled neurons and the number of labeled medial (M) OC neurons, suggesting that the spread of labeling proceeded mainly via synapses on MOC neurons. In the cochlear nucleus, the transneuronally labeled neurons were seen as being multipolar cells. Transneuronal labeling was also observed in the central nucleus of the inferior colliculus, in the auditory cortex and in centers not typically associated with the auditory pathway such as the pontine reticular formation, subcoerulean nucleus, and the pontine dorsal raphe. These non-auditory inputs represent important pathways to OC neurons, but their functional role in the reflex pathways and in hearing remains to be clarified. However, transneuronal labeling that spread further up the chains of the reflex into the inferior colliculus and cortex was more indistinct and more difficult to analyze due to the cytopathic and degenerative elements of the viral protein. Improvements in the labeling properties of the viral tracer should help to eliminate these problems thus allowing the research community to expand their search for sources of influence on the auditory periphery. It is likely that the labeling technology will improve substantially over the next few years in terms of longevity, minimal cytopathic damage, practical aspects in preservation and administration. Further understanding of the auditory peripheral reflex pathways will increase exponentially so as to further understand a complex characteristic in humans. A highly complicated middle ear and brainstem structure has baffled scientists and clinicians since the famous and revered Fabricius first sat down and theorized it's precise function in humans. This is a structure that warrants further study to explore the intricate inter-neurons within the main components of the reflex pathway that is responsible for it's activation by higher neural, auditory and non auditory inputs.

13. REFERENCES

Adams, J. C. (1983). Multipolar cells in the ventral cochlear nucleus project to the dorsal cochlear nucleus and the inferior colliculus. *Neuroscience Letters, 37*, 205-208.

Adams, J. C. (1986). Neuronal morphology in the human cochlear nucleus. *Archives of Otolaryngology: Head and Neck Surgery, 112*, 1253-1261.

Adams, J. C. (1989). Functional study of the ventral cochlear nucleus using immunocytochemistry. *Association for Research in Otolaryngology Abstracts, 12*, 3.

Aiken SJ, Andrus JN, Bance M, Phillips DP (2013) Acoustic stapedius reflex function in man revisited. *Ear Hear* Jul-August; 34(4): e38-51

Anderson, H., Barr, B., & Wedenberg, E. (1969a). Early diagnosis of 8th-nerve tumours by acoustic reflex tests. *Acta Oto-Laryngologica Supplementum, 263*, 232-237.

Anderson, H., Barr, B., & Wedenberg, E. (1969b). Intra-aural reflexes in retrocochlear lesions. In C. A. Hamberger & J. Wersall (Eds.), *Disorders of the skull base region* (pp. 48-54). Stockholm, Sweden: Almquvist & Wikell.

Anderson, H., & Barr, B. (1971). Conductive high tone hearing loss. *Archives of Otolaryngology, 93*, 599-605.

Anderson, H. C., Jepsen, O., & Ratjen, E. (1962). Ossicular-chain defects: Diagnosis and treatment. *Acta Oto-Laryngologica, 54*, 393-402.

Andrade KC, Leal Mde C, Muniz LF, Menezes Pde, Albuquerque KM, Carnauba AT (2014). The importance of electrically evoked stapedial reflex in cochlear implant. *Braz J Otorhinolaryngol*. Jan-Feb;80(1):68-77

Anniko, M., & Wroblewski, R. (1981). Ultrastructure and elemental composition of the stapedius muscle in guinea pig. *European Archives of Oto-Rhino-Laryngology, 230*, 109-112.

Apostolides PF, Trussell LO (2014). Superficial stellate cells of the dorsal cochlear nucleus. *Front Neural Circuits.* 2014 Jun 10;8:63

Aschoff, A., Ostwald, J. (1987). Different origins of cochlear efferents in some bat species, rats and guinea pigs. *J. Comp. Neurol. 264*, 56–72.

Avan, P., Loth, D. (1992). Hypothetical roles of middle ear muscles in guinea-pig. *Hear. Res. 59*, 59–69.

Azeredo, W., Woods, C., Sterns, A., & Relkin, E. (2000). Temporal characteristics of DPOAE suppression with auditory brainstem stimulation. *Association for Research in Otolaryngology Abstracts, 23*, 139.

Badia L, Parikh A, Brookes GB. Management of middle ear myoclonus. *J Laryngol Otol*. 1994;108:380-382.

Baust, W., Berlucchi, G., & Moruzzi, C. (1964). Change in the auditory input during arousal in cats with tenotomised middle ear muscles. *Archives Italiennes de Biologie, 102*, 675-685.
Benson, T. E., & Brown, M. C. (2006). Ultrastructure of synaptic input to medial olivocochlear neurons. *Journal of Comparative Neurology, 499*, 244-257.
Benson, T. E., Brown, M. C., & Lee, D. J. (2008). Ultrastructural features of inputs to tensor tympani motoneurons in the rat.
Benson TE, Lee DJ, Brown MC (2013). Tensor tympani motoneurons receive mostly excitatory synaptic inputs. *Anat Rec (Hoboken).* Jan;296(1):133-45.
Association for Research in Otolaryngology Abstracts No. 552.
Bento RF, Sanchez TG, Miniti A, et al. Continuous, high frequency objective tinnitus caused by middle ear myoclonus. *Ear Nose Throat J*. 1998;77:814-818.
Bergquist, F., & Ludwig, M. (2008). Dendritic transmitter release: a comparison of two model systems. *Journal of Neuroendocrinology, 20*, 677-686.
Berkowitz, L. A., Riabowol, K. T., & Gilman, M. Z. (1989). Multiple sequence elements of a single functional class are required for cyclic AMP responsiveness of the mouse c-fos promoter. *Molecular and Cellular Biology, 9*, 4272-4281.
Berkowitz, A., Yosten, G.L., Ballard, R.M. (2006). Somato-dendritic morphology predicts physiology for neurons that contribute to several kinds of limbmovements. *J. Neurophysiol. 95*, 2821–2831.
Bess, H. F., & Humes, L. E. (2008). *Audiology: The fundamentals* (4th ed.). Philadelphia, PA: Lippincott Williams & Wilkins.
Bhimrao et al., (2012). "Systematic review of management strategies for middle ear myoclonus." Otolaryngology - Head & Neck Surgery 146(5): 698-706.
Billig, I., Yeager, M. S., Blikas, A., & Raz, Y. (2007). Neurons in the cochlear nuclei controlling the tensor tympani muscle in the rat: A study using pseudorabies virus. *Brain Research, 1154*, 124-136.
Bird, S., Gulley, R., Wenthold, R., & Fex, J. (1978). Kainic acid injections result in degeneration of cochlear nucleus cells innervated by the auditory nerve. *Science, 202*, 1087-1089.
Biswas D, Mal RK (2013). Absent stapedial reflex: otosclerosis or middle ear tumor? *Ear Nose Throat J.* 2013 Feb;92(2)
Blanke A, Aupperle H, Seeger J, Kubick C, Schusser GF. (2014). Histological study of the external, middle and inner ear of horses. *Anat Histol Embryol*. Oct 6
Blevins, C. E. (1964). Studies on the innervation of the stapedius muscle of the cat. *Anatomical Record, 149*, 157-172.
Boldogkoi, Z., Bratincsak, A., & Fodor, I. (2002). Evaluation of pseudorabies virus as a gene transfer vector and an oncolytic agent for human tumor cells. *Anticancer Research, 22*, 2153-2159.
Bordure, P., O'Donoghue, G. M., & Mason, S. (1996). Electrophysiologic and other objective tests in pediatric cochlear implantation. *Annales D'Otolaryngologieet de Chirurgie Cervico Faciale, 113*,

147-154.
Borg, E., Moller, A.R. (1968). The acoustic middle ear reflex in unanesthetized rabbits. *Acta Otolaryngol. 65* (6), 575–585.
Borg, E. (1971). Efferent inhibition of afferent acoustic activity in the unanesthetized rabbit. *Experimental Neurology, 31*, 301-312.
Borg, E. (1972). On the use of acoustic middle ear muscle reflexes in studies of auditory function in nonanesthetized rabbits. *Acta Oto-Laryngologica*, *74*, 240-247.
Borg, E. (1973). On the neuronal organization of the acoustic middle ear reflex: A physiological and anatomical study. *Brain Research, 49*, 101-123.
Borg, E., & Moller, A. R. (1975). Effect of central depressants on the acoustic middle ear reflex in rabbit. *Acta Physiologica Scandinavica, 94*, 327-338.
Borg, E., & Zakrisson, J. E. (1974). Stapedius reflex and monaural masking. *Acta Oto-Laryngologica*, *78*, 155-161.
Borg, E., & Zakrisson, J. E. (1975). The activity of the stapedius muscle in man during vocalization. *Acta Oto-Laryngologica, 79*(5-6), 325-333.
Borg, E., Counter, S. A., & Rosler, G. (1984). Theories of middle ear muscle functions. In S. Silman (Ed.), *The acoustic reflex: Basic principles and clinical applications* (pp. 63-101). New York, NY: Academic Press.
Borg, E., Nilsson, R., & Engstrom, B. (1983). Effect of the acoustic reflex on inner ear damage induced by industrial noise. *Acta Oto-Laryngologica, 96*(5-6), 361-369.
Brask, T. (1979). The noise protection effect of the stapedius reflex. *Acta Otolaryngol. Suppl. 360*, 116–117.
Brawer, J. R., Morest, D. K., & Kane, E. C. (1974). The neuronal architecture of the cochlear nucleus of the cat. *Journal of Comparative Neurology, 155*, 251-300.
Brosch S, Riechelmann H, Johannsen HS. Myoclonus of the middle ear: a rare, differential diagnosis for objective tinnitus [in German]. *HNO*. 2003;51:421-423.
Brown MC, de Venecia RK, Guinan JJ, Jr (2003) Responses of medial olivocochlear (MOC)neurons: Specifying the central pathways of the MOC reflex. Exp Brain Res 153**:**491- 498
Brown, M. C., & Levine, J. L. (2008). Dendrites of medial olivocochlear neurons in mouse. *Neuroscience, 154*, 147-159.
Christian Brown M, Lee DJ, Benson TE. (2013). Ultrastructure of spines and associated terminals on brainstem neurons controlling auditory input. *Brain Re*. 2013 Jun 21; 1516:1-10
Callan, D. E., Lasky, R. E., & Fowler, C. G. (1999). Neural networks applied to retrocochlear diagnosis. *Journal of Speech, Language, and Hearing Research, 42*, 287-299.
Campbell, J.P., Henson, M.M. (1988). Olivocochlear neurons in the brainstem of the mouse. *Hear. Res. 35,* 271–274.

Caner, G., Olgun, L., Gultekin, G., & Balaban, M. (2007). Optimizing fitting in children using objective measures such as neural response imaging and electrically produced reflex threshold. *Otology & Neurotology, 28*, 637-640.
Cant, N. B. (1982). Identification of cell types in the anteroventral cochlear nucleus that project to the inferior colliculus. *Neuroscience Letters, 32*, 241-246.
Card, J. P. (2001). Pseudorabies virus neuroinvasiveness: A window into the functional organization of the brain. *Advances in Virus Research, 56*, 39-71.
Carmel, P. W., & Starr, A. (1963). Acoustic and non-acoustic factors modifying middle-ear muscle activity in waking cats. *Journal of Neurophysiology, 26*, 598-616.
Chien, W., & Lee, D. J. (2009). Physiology of the auditory brainstem. In P. W. Flint, C. W. Cummings, D. E. Schuller, & J. R. Thomas (Eds.), *Otolaryngology: Head and neck surgery* (5th ed.). (pp.1838-1849) Amsterdam, Netherlands: Elsevier Health Sciences.
Chiveralls, K., Fitzsimmons, R., Beck, G. B., & Kernohan, H. (1976). The diagnostic significance of the acoustic reflex. *British Journal of Audiology, 10*, 122-128.
Cohen, E. S., Brawer, J. R., & Morest, D. K. (1972). Projections of the cochlea to the dorsal cochlear nucleus in the cat. *Experimental Neurology, 5*, 470-479.
Cohen D, Perez R. Bilateral myoclonus of the tensor tympani: a case report. *Otolaryngol Head Neck Surg*. 2003;128:441.
Colletti, L.; Shannon, R.; Colletti, V. (2012). "Auditory brainstem implants for neurofibromatosis type 2.". *Curr Opin Otolaryngol Head Neck Surg* **20** (5): 353–7.
Colletti, V.; Carner, M.; Miorelli, V.; Guida, M.; Colletti, L.; Fiorino, F. (2005). "Auditory brainstem implant (ABI): new frontiers in adults and children.". *Otolaryngol Head Neck Surg* **133** (1): 126–38.
Conradi, S., Kellerth, J. O., Berthold, C. H., & Hammarberg, C. (1979). Electron microscopic studies of serially sectioned cat spinal alpha-motoneurons. IV. Motoneurons innervating slowtwitch (type S) units of the soleus muscle. *Journal of Comparative Neurology, 184*, 769-782.
Contretas RJ, Gomez MM, Norgren R (1980) Central origins of cranial nerve parasympathetic neurons in the rat. J Comp Neurol 190:373–394.
Counter, S. A. (2007). Brainstem mediation of the stapedius muscle reflex in hydranencephaly. *Acta Oto-Laryngologica*, *127*, 498-504.
Courville, J. (1966). Rubrobulbar fibers to the facial nucleus and the lateral reticular nucleus (nucleus of the lateral funiculus). An experimental study in the cat with silver impregnation methods. *Brain Research, 1*, 317-337.
Coyle, J. T., Molliver, M. E., & Kuhar, M. J. (1978). In situ injection of kainic acid: a new method for selectively lesioning neural cell

bodies while sparing axons of passage. *Journal of Comparative Neurology, 180*, 301-323.
Cunningham, A. (1985). *Fabricius and the Aristotle project: Anatomical teaching and research at Padova*. Cambridge, England: Cambridge University Press.
Cunningham A. Fabricius and the Aristotle Project. Anatomical teaching and research at Padova. In: French RK, Lonie IM, Wear A, eds. The medical renaissance of the sixteenth century. Cambridge: Cambridge University Press,1985;195-222
Dammeijer, P. F., van Dijk, P., Manni, J. J., & van Mameren, H. (2006). Stapedius muscle fiber characterization during postnatal development in the rat. *Hearing Research, 219*(1-2), 48-55.
Darrow KN, Maison SF, Liberman MC (2006) Cochlear efferent feedback balances interaural sensitivity. Nat Neurosci 9:1474-1476
Darrow KN, Drottar M, Brown MC (2012) Planar multipolar cells in the cochlear nucleus project
to medial olivocochlear neurons in mouse. J Comp Neurol 520:1365-1375
Delano P, Elgueda D, Hamame C, Robles L (2007) Selective attention to visual stimuli reduces cochlear sensitivity in chinchillas. J Neurosci 27:4146-4153
de Venecia, R. K., Liberman, M. C., Guinan, J. J., Jr., & Brown, M. C. (2005). Medial olivocochlear reflex interneurons are located in the posteroventral cochlear nucleus: A kainic acid lesion study in guinea pigs. *Journal of Comparative Neurology, 487*, 345-360.
Djupesland, G. (1964). Middle-ear muscle reflexes elicited by acoustic and nonacoustic stimulation. *Acta Oto-Laryngologica Supplementum, 188*, 287-292.
Djupesland, G. (1967). *Contractions of the tympanic muscles in man*. Oslo, Norway: Universitetsforlaget Oslo.
Djupesland, G. (1976). Nonacoustic reflex measurement procedures, interpretations and variables. In A. S. W. Feldman (Ed.), *Acoustic impedance and admittance: The measurement of middle ear function* (pp. 217-235). Baltimore, MD: Williams & Wilkins.
Donga, R., Dubuc, R., Kolta, A., & Lund, J. P. (1992). Evidence that the masticatory muscles receive a direct innervation from cell group k in the rabbit. *Neuroscience, 49*, 951-961.
Doucet, J. R., Ross, A. T., Gillespie, M. B., & Ryugo, D. K. (1999). Glycine immunoreactivity of multipolar neurons in the ventral cochlear nucleus which project to the dorsal cochlear nucleus. *Journal of Comparative Neurology, 408*, 515-531.
Doucet, J. R., & Ryugo, D. K. (1997). Projections from the venteral cochlear nucleus to the dorsal cochlear nucleus in rats. *Journal of Comparative Neurology, 385*, 245-264.
Doyle, W. J., & Rood, S. R. (1980). Comparison of the anatomy of the eustachian tube in the rhesus monkey (*Macaca mulatta*) and man: Implications for physiologic modeling. *Annals of Otology, Rhinology, and Laryngology, 89*, 49-57.
Ebert, C. S., Zanation, A. M., & Buchman, C. A. (2008). Another

cause for conductive hearing loss with present acoustic reflexes. *Laryngoscope, 118*, 2059-2061.
Duverney GJ. “Contenant la structure, les ufages & les maladies de toutes les parties de l’orielle”. In: "Traite de l'organe de l'ouie. Paris: Chez Estienne Michallet, 1683: Planche 6-7
Ehret, G. (1979). Quantitative analysis of nerve fibre densities in the cochlear of the house mouse (Mus musculus). *J. Comp. Neurol. 183,* 73–88.
Ekstrand MI, Enquist LW, Pomeranz LE (2008) The alpha-herpesviruses: Molecular pathfinders in nervous system circuits. Trends Mol Med 14:134-140
Enquist LW, Card JP (2003) Recent advances in the use of neurotropic viruses for circuit analysis. Curr Opin Neurobiol 13:603-606
Eustachii, B. (1564). Bartholomaei Eustachii Sanctoseverinatis medici ac philosophi Opuscula anatomica. Quorum numerum & argumenta aversa pagina indicabit, Vol., Vincentius Luchinus excudebat, Venetiis.
Eusthaci B. Bartholomaei Eustachii Sanctoseverinatis medici ac philosophi Opuscula anatomica. Quorum numerum & argumenta aversa pagina indicabit. Venetiis: Vincentius Luchinus excudebat,1564;158-162
Evans, E F., & Nelson, P. G. (1973). On the functional relationship between the dorsal and ventral subdivisions of the cochlear nucleus of the cat. *Experimental Brain Research, 17*, 428-442.
Fabrici ab Acquapendente H. De Visione, Voce et Auditu.Venice, F. Bolzettam, 1600.
Faye-Lund H (1986) Projection from the inferior colliculus to the superior olivary complex in the albino rat. Anat Embryol 175:35-52
Fay, R.R. (1988). Hearing in Vertebrates: *a Psychophysics Databook*. Heffernan Press, Worcester, MA.
Fee, W. E. J., Dirks, D. D., & Morgan, D. E. (1975). Nonacoustic stimulation of the middle ear muscle reflex. *Annals of Otology, Rhinology, and Laryngology, 84*, 80-87.
Fekete, D. M. (1984). *The central projections of auditory nerve fibers in cats*. (Unpublished doctoral dissertation). Harvard University, Cambridge, MA.
Fekete, D. M., Rouiller, E. M., Liberman, M. C., & Ryugo, D. K. (1984). The central projections of intracellularly labeled auditory nerve fibers in cats. *Journal of Comparative Neurology, 229*, 432-450.
Finger S. Origins of neuroscience: A history of explorations into brain function. New York: Oxford University Press US, 2001:108-88.
Fisher, G. L., & Harrison, J. M. (1962). Some functions of the superior olivary complex in auditory intesity discrimination. *Journal of Comparative Neurology, 119*, 269-279.
Fitzgerald, J. T., Gruener, G., & Mtui, E. (2007). *Clinical neuroanatomy and neuroscience*. Amsterdam, Netherlands: Elsevier.
Friauf, E., & Baker, R. (1985). An intracellular HRP-study of cat tensor tympani motoneurons. *Experimental Brain Research, 57*, 499-511.

Frijns JH, de Snoo SL, Kate JH (1996). Hear Res. 1996 May; 95(1-2):33-48.
Friauf, E., & Ostwald, J. (1988). Divergent projections of physiologically characterized rat ventral cochlear nucleus neurons as shown by intra-axonal injection of horseradish peroxidase. *Experimental Brain Research, 73*, 263-284.
Gacek, R.R., Rasmussen,G.L. (1961). Fiber analysis of the statoacoustic nerve of guinea pig, cat, and monkey. *Anat. Rec. 139*, 455–463.
Geiringer, E. (1970). The rebel and the conservative in the history of medicine. *New Zealand Medical Journal, 71*, 370-375.
Gelfand, S. A. (1984). The contralateral acoustic reflex. In S. Silman (Ed.), *The acoustic reflex: Basic principles and clinical applications* (pp. 137-186). New York, NY: Academic Press.
Gelfand, S. A. (1998). *Hearing: An introduction to psychological and physiological acoustics*. New York, NY: Marcel Dekker.
Girardet, R. (1960). Contribution to the study of the innervation of the tensor tympani. *Archives D'anatomie, D'histologie et D'embryologie Normales et Expérimentales, 43*, 121-164.
Gironell, A., Kulisevsky, J., Roig, C., Pascual-Sedano, B., Rodriguez-Fornells, A., & Otermin, P. (2003). Diagnostic potential of acoustic startle reflex, acoustic blink reflex, and electro-oculography in progressive supranuclear palsy: A prospective study. *Movement Disorders, 18*, 1273-1279.
Godfrey DA, Kiang NYS, Norris BE (1975) Single unit activity in the posteroventral cochlear nucleus of the cat. J Comp Neurol 162:247-268
Groff A, Liberman MC (2003) Modulation of cochlear afferent response by the lateral olivocochlear system: activation via electrical stimulation of the inferior colliculus. J Neurophysiol 90:3178–3200
Guinan, J. J., Jr., Joseph, M. P., & Norris, B. E. (1989). Brainstem facialmotor pathways from two distinct groups of stapedius motoneurons in the cat. *Journal of Comparative Neurology, 287*, 134-144.
Ha CK. Objective ear click of middle-ear myoclonus induced by a welding flux into ear canal. *Parkinsonism Relat Disord*. 2007;1.307:S78.
Hackney CM, Osen KK, Kolston J (1990) Anatomy of the cochlear nuclear complex of the guinea pig. Anat Embryol 182:123–149
Haines, D. E., & Lancon, J. A. (2003). *Neuroanatomy*. New York, NY. Lippincott Williams & Wilkins.
Harrison, J. M., & Warr, W. B. (1962). A study of the cochlear nuclei and the ascending auditory pathways of the medulla. *Journal of Comparative Neurology, 119*, 341-380.
Harrison, J. M., & Feldman, M. L. (1970). Anatomical aspects of the cochlear nucleus and superior olivary complex. In W. D. Neff (Ed.), *Contributions to sensory physiology* (pp. 95-142). New York, NY: Springer-Verlag.
Heaviside O. Electrical papers by Oliver Heaviside (in two volumes). New York and London: MacMillon & Co, 1894:268 (article 26).

Held, H. (1893). Die centrale Gehörleitung. *Archiv für Anatomie und Physiologie, 17*, 201-248.
Helfferich, F., Uhereczky, G., Boldogkoi, Z., Vitez, L. C., & Palkovits, M. (2003). Investigation of the complex descending innervation of the dorsal cochlear nucleus in the rat: A transneuronal tract-tracing study using pseudorabies virus. *Neuroscience Letters, 337*, 151-154.
Hensen, V. (1878). Beobachtungen uber die Thatigkeit des Trommelfell-spanners bei Hund und Katze. *Archiv fur Anatomie und Physiologie, Physiologische Abtheilung, 2*, 312-319.
Herrmann BS, Brown MC, Eddington DK, Hancock KE, Lee DJ (2015). Auditory Brainstem Implants: Eletrophysiologic responses and subject perception. Ear Hear. May-Jun;36(3):368-76.
Hieronymus Fabricius ab Aquapendente (1533-1620). In: Zimmerman L, Veith I, eds. Great ideas in the history of surgery. San Francisco: Norman Publishing, 1993;230-34.
Hickmott, P. W., & Ethell, I. M. (2006). Dendritic plasticity in the adult neocortex. *Neuroscience, 12*, 16-28.
Hiroshi H, Youhei H, Jun O, Shigeki G, Tetsuaki K & Toshimitsu K (2013). Middle ear myoclonus cured by selective tenotomy of the tensor tympani: Strategies for targeted intervention for middle ear muscles. *Otology & Neurotology*, 34: 1152-1558
Hodges, A., Balkany, T., Ruth, R., Lambert, P., Dolan-Ash, S., & Schloffman J. (1997). Electrical middle ear muscle reflex: Use in cochlear implant programming. *Otolaryngology-Head and Neck Surgery, 117*, 255-261.
Horvath, M., Kraus, K.S., Illing, R.B., 2000. Olivocochlear neurons sending axon collaterals into the ventral cochlear nucleus of the rat. J. Comp. Neurol. 422, 95–105.
Horvath M, Ribari O, Repassy G, Toth IE, Boldogkoi Z, Palkovits M (2003) Intracochlear injection of pseudorabies virus labels descending auditory and monoaminerg projections to olivocochlear cells in guinea pig. Eur J Neurosci 18:1439-1447
House WF, Hitselberger WE (Feb 2001). "Twenty-year report of the first auditory brain stem nucleus implant".
Hunter, L. L., Ries, D. T., Schlauch, R. S., Levine, S. C., & Ward, W. D. (1999). Safety and clinical performance of acoustic reflex tests. *Ear and Hearing, 20*, 506-514.
Hurley PA, Clarke M, Crook JM, Wis AK, Shepherd RK Cochlear immunochemistry—a new technique based on gelatin embedding. J Neurosci Meth 129:81–86
Hutson, K. A., Glendenning, K. K., & Masterton, R. B. (1979). Accessory abducens nucleus and its relationship to the accessory facial and posterior trigeminal nuclei in cat. *Journal of Comparative Neurology, 188*, 1-16.
Immittance/Impedance Measurement. Available at: http://www.bsure4balance.com/about_us/heritage/story_of_gn_otometrics_backtround/story_of_gn_otometrics_backtround_immittance.htm. Accessed March 2008
Ikiz, A. O., Unsal, E., Kirkim, G., Erdag, T. K., & Guneri, E. A.

(2007). Hearing loss and middle ear involvement in patients with juvenile idiopathic arthritis. *International Journal of Pediatric Otorhinolaryngology, 71*, 1079-1085.
Ito, J., & Honjo, I. (1988). Electrophysiological and HRP studies of the direct afferent inputs from the cochlear nuclei to the tensor tympani muscle motoneurons in the cat. *Acta Oto-Laryngologica, 105*, 292-296.
Itoh, K., Kamiya, H., Mitani, A., Yasui, Y., Takada, M., & Mizuno, N. (1987). Direct projections from the dorsal column nuclei and the spinal trigeminal nuclei to the cochlear nuclei in the cat. *Brain Research, 400*, 145-150.
Itoh, K., Nomura, S., Konishi, A., Yasui, Y., Sugimoto, T., & Muzino, N. (1986). A morphological evidence of direct connections from the cochlear nuclei to tensor tympani motoneurons in the cat: A possible afferent limb of the acoustic middle ear reflex pathways. *Brain Research, 375*, 214-219.
Izzo AD, Richter CP, Jansen ED, Walsh JT (2006). Laser stimulation of the auditory nerve. Lasers in Surg. Med. 38: 745-753.
Jean-Baptiste, M., & Morest, D. K. (1975). Transneuronal changes of synaptic endings and nuclear chromatin in the trapeziod body following cochlear ablations in cats. *Journal of Comparative Neurology, 162*, 111-134.
Jepsen O. The acoustic stapedius reflex in man. Measurements of the acoustic impedance of the tympanic membrane in normal individuals and in patients with peripheral facial paralysis. Academietsuniversitetet. Aarhus, Denmark, 1955.
Jerger, J., Harford, E., & Clemis, J. (1974). Studies in impedance audiometry III. Middle ear disorders. *Archives of Otolaryngology, 99*, 165-171.
Jerger, J., & Hayes, D. (1983). Latency of the acoustic reflex in eighth-nerve tumor. *Archives of Otolaryngology, 109*, 1-5.
Jerger, J., & Jerger, S. (1975). Clinical validity of central auditory tests. *Scandinavian Audiology, 4*, 147-163.
Jerger, S., Jerger, J., & Hall, J. (1979). A new acoustic reflex pattern. *Archives of Otolaryngology, 105*, 24-28.
Jerger, S. (1980). Diagnostic application of impedance audiometry in central auditory disorders. In J. Jerger, & J. L. Northern (Eds.), *Clinical impedance audiometry* (pp. 128-140). Acton, England: American Electromedics.
Jones SE, Mason MJ, Sunkaraneni VS, Baguley DM (2008). The effect of auditory **stimulation** on the **tensor tympani** in patients following stapedectomy. *Acta Otolaryngol*. 2008 Mar;128(3):250-4.
Joseph, M. P., Guinan, J. J. Jr., Fullerton, B. C., Norris, B. E., & Kiang, N. Y. (1985). Number and distribution of stapedius motoneurons in cats. *Journal of Comparative Neurology, 232*, 43-54.
Kato, T. (1913). Zur Physiologie der Binnenmuskeln des Ohres.

Archiv fur die gesamte Physiologie des Mehschen und der Tiere, 150, 569-573.
Keithley, E.M., Feldman, M.L. (1979). Spiral ganglion cell counts in an age-graded series of rat cochleas. *J. Comp. Neurol. 188*, 429–442.
Keller, J.T., Saunders, M.C., Ongkiko, C.M., Johnson, J., Frank, E., Van Loveren, H., Tew Jr, J.M. (1983). Identification of motoneurons innervating the tensor tympani and tensor veli palatini muscles in the cat. *Brain Res. 270*, 209–215.
Kelly, J. B., van Adel, B. A., & Ito, M. (2009). Anatomical projections of the nuclei of the lateral lemniscus in the albino rat (Rattus norvegicus). *Journal of Comparative Neurology, 512*, 573-593.
Kemp, D. T., & Chum, R. (1980). Properties of the generator of stimulated acoustic emissions. *Hearing Research, 2*(3-4), 213-232.
Kiang, N. Y-S., Morest, D. K., Godfrey, D. A., Guinan, J. J., Jr., & Kane, E. C. (1973). Stimulus coding at caudal levels of the cat's auditory nervous system; Response characteristics of single units. In A. R. Moller (Ed.), *Basic mechanisms of hearing* (pp. 455-478). New York, NY: Academic Press.
Kierner, A. C., Mayer, R., & v. Kirschhofer, K. (2002). Do the tensor tympani and tensor veli palatini muscles of man form a functional unit? A histochemical investigation of their putative connections. *Hearing Research, 165*, 48-52.
Kita, H., Chiken, S., Tachibana, Y., & Nambu, A. (2007). Serotonin modulates pallidal neuronal activity in the awake monkey. *Journal of Neuroscience, 27*, 75-83.
188 *Trends in Amplification 14(3)*
Klockhoff, I. (1961). Middle ear reflexes in man. *Acta Oto-Laryngologica Supplementum, 164*, 1-92.
Klockhoff, I., Anderson, H. (1960). Reflex activity in the tensor tympani muscle recorded in man; preliminary report. *Acta Otolaryngol. 51,* 184–188.
Klockhoff, I., & Anderson, H. (1960). Reflex activity in the tensor tympani muscle recorded in man: Preliminary report. *Acta Oto-Laryngologica, 51*, 184-188.
Kulesza RJ Jr (2014) Characterization of human auditory brainstem circuits by calcium-binding protein immunohistochemistry *Neuroscience.* Jan 31;258:318-31
Lee, D.J., Brown, M.C., Benson, T.E. (2009). Ultrastructure features of inputs to tensor tympani motoneurons in rats. *Assoc. Res. Otolaryngol.* (Abstract # 552).
Lee, D. J., Benson, T. E., & Brown, M. C. (2008). Diverse synaptic terminals on rat stapedius motoneurons. *Journal of the Association for Research in Otolaryngology, 9*, 321-333.
Lee, D. J., Brown, M. C., & Benson, T. E. (2009). Synaptic terminals on middle ear muscle motoneurons: Vesicle morphometry. *Association for Research in Otolaryngology,* Abstract No. 640.
Lee, D. J., de Venecia, R. K., Guinan, J. J., Jr., & Brown, M. C.

(2006). Central auditory pathways mediating the rat middle ear muscle reflexes. *Anatomical Record, 288*, 358-369.
Lee, Y., Lopez, D. E., Meloni, E. G., & Davis, M. (1996). A primary acoustic startle pathway: Obligatory role of cochlear root neurons and the nucleus reticularis pontis caudalis. *Journal of Neuroscience, 16*, 3775-3789.
Lenarz, T et al. (2001). "Auditory brainstem implant: part I. Auditory performance and its evolution over time". *Otol Neurotol* **22** (22): 823–833.
Liberman MC, Brown MC (1986). Physiology and anatomy of single olivocochlear neurons in the cat. Hearing Res 24:17-36.
Liberman, M. C. (1991). Central projections of auditory-nerve fibers of differing spontaneous rates. I. Anteroventral cochlear nucleus. *Journal of Comparative Neurology, 313*, 240-258.
Liberman, M. C., & Guinan, J. J., Jr. (1998). Feedback control of the auditory periphery: Anti-masking effects of middle ear muscles vs. olivocochlear efferents. *Journal of Communication Disorders, 31*, 471-483.
Lilly, D. J. (1972). Acoustic impedance at the tympanic membrane. In J. Katz (Ed.), *Handbook of clinical audiology* (pp. 434-469). Baltimore, MD. Williams & Wilkins.
Liu HB, Fan JP, Lin SZ, et al. Botox transient treatment of tinnitus due to stapedius myoclonus: case report. *Clin Neurol Neurosurg*. 2011;113:57-58.
Loewy, A. D. (1998). Viruses as transneuronal tracers for defining neural circuits. *Neuroscience & Biobehavioral Reviews, 22*, 679-684.
Loftus WC, Malmierca MS, Bishop DC, Oliver DL (2008) The cytoarchitecture of the inferior colliculus revisited: a common organization of the lateral cortex in rat and cat. Neuroscience 154:196–205
Luascher, E. (1930). Die Funktion des Musculus stapedius beim Menschen. II. Teil. *Zeitschrift fur Hals-, Nasen- und Ohrenheilkunde, 25*, 462-478.
Lyon, M.J. (1975). Localization of the efferent neurons of the tensor tympani muscle of the newborn kitten using horseradish peroxidase. *Exp. Neurol. 49*, 439–455.
Lyon, M. J. (1978). The central location of the motor neurons to the stapedius muscle in the cat. *Brain Research, 143*, 437-444.
Lyon, M. J., & Malmgren, L. T. (1982). A histochemical characterisation of muscle fiber types in the middle ear muscles of the cat. I. The stapedius muscle. *Acta Oto-Laryngologica, 94*(1-2), 99-109.
Lyon, M. J., & Malmgren, L. T. (1988). Muscle fiber types in the cat midle ear muscles. II. Tensor Tympani. *Archives of Otolaryngology, 114*, 404-409.
Mahoney, T., Vernon, J., & Meikle, M. (1979). Function of the acoustic reflex in discrimination of intense speech. *Archives of Otolaryngology, 105*, 119-123.

Masterton, R. B., & Granger, E. M. (1988). Role of the acoustic striae in hearing: Contribution of dorsal and intermediate striae to detection of noises and tones. *Journal of Neurophysiology, 60*, 1841-1860.
Maurizi, M., Ottaviani, F., Paludetti, G., & Lungarott, I. S. (1985). Ossicular chain interruption with present acoustic reflex. *International Journal of Pediatric Otorhinolaryngology, 9*, 227-232.
McCue, M. P., & Guinan, J. J., Jr. (1988). Anatomical and functional segregation in the stapedius motoneuron pool of the cat. *Journal of Neurophysiology, 60*, 1160-1180.
McGeer, P. L., & McGeer, E. G. (1978). Kainic acid as a tool in neurobiology. In J. W. McGeer, J. W. Olney, & P. L. McGeer (Eds.), *Kainic acid as a tool in neurobiology* (pp. 123-138). New York, NY: Raven Press.
Melcher, J. R., & Kiang, N. Y. (1996). Generators of the brainstem auditory produced potential in cat. III Identified cell populations. *Hearing Research, 93*, 52-71.
Mesulam MM (1982) Principles of horseradish peroxidase neurohistochemistry and their applications for tracing neural pathways—axonal transport, enzyme histochemistry and light microscopic analysis. In: Mesulam M-M (ed) Tracing neural connections with horseradish peroxidase. Wiley, Chichester, pp 1–151
Metz, O. (1946). The acoustic impedance measured on normal and pathological ears. Orientating studies on the applicability of impedance measurements in otological diagnosis (Unpublished doctoral dissertation). University of Copenhagen, Copenhagen, Denmark.
Metz, O. (1952). Threshold of reflex contractions of muscles of middle ear and recruitment of loudness. *Acta Oto-Laryngologica, 55*, 536-543.
Mizuno, N., Nomura, S., Konishi, A., Uemura-Sumi, M., Takahashi, O., Yasui, Y., Matsushima R. (1982). Localization of motoneurons innervating the tensor tympani muscles: An horseradish peroxidase study in the guinea pig and cat. *Neuroscience Letters, 31*, 205-208.
Moller, A. R. (1974). Responses of units in the cochlear nucleus to sinusoidally amplitude-modulated tones. *Experimental Neurology, 45*, 104-117.
Moller, A. R. (1984). Neurophysiological basis of the acoustic middle-ear reflex. In S. Silman (Ed.), *The acoustic reflex: Basic principles and clinical applications* (pp. 1-35). New York, NY: Academic Press.
Moller, A. R. (2006). *Hearing: Anatomy, physiology and disorders of the auditory system*. St. Louis, MO: Academic Press.
Mudry A. Guichard Joseph Duverney (1648-1730), le premier otologiste Français au 17ème siècle. Ann Otolaryngol Chir Cervicofac 2000 Sep;117 (4):203-9.

Mukerji, S., Brown, M. C., & Lee, D. J. (2009). A morphologic study of fluorogold labeled tensor tympani motoneurons in mice. *Brain Research, 1278*(30), 59-65.
Mukerji, S., & Lee, D. J. (2010). De visione, voce et auditu: The contribution of Hieronymous Fabricius to our understanding of tensor tympani function. *Otology & Neurotology, 31*, 536-543.
Mukerji, S., & Lee, D.J (2009). Optical stimulation of the central nervous system (Poster presentation: Annual ARO Meeting 2009 (Baltimore, Maryland).
Mulders WHAM, Robertson D (2000a) Evidence for direct cortical innervation of medial olivocochlear neurones in rats. Hearing Res 144:65-72
Mulders WHAM, Robertson D (2000b) Morphological relationships of peptidergic and noradrenergic nerve terminals to olivocochlear neurones in the rat. Hearing Res. 144: 53-64
Mulders WHAM, Robertson D (2001) Origin of the noradrenergic innervation of the superior olivary complex in the rat. J. Chem. Neuroanat. 21: 313-322.
Mulders WHAM, Robertson D (2002) Inputs from the cochlea and the inferior colliculus converge on olivocochlear neurons. Hearing Res 167:206-213
Mulders WHAM, Robertson D (2005a) Noradrenergic modulation of brainstem nuclei alters cochlear neural output. Hearing Res 204:147-155
Mulders WHAM, Robertson D (2005b) Catecholaminergic innervation of guinea pig superior olivary complex. Chem. Neuroanat. 30:230-242
Murata, K., Ito, S., Horikawa, J., & Minami, S. (1986). The acoustic middle ear muscle reflex in albino rats. *Hearing Research, 23*, 169-183.
Nahin PJ. Oliver Heaviside: Sage in Solitude-The life, work and times of an electrical genius of the Victorian age. New York: The institute of electrical and electronics engineers Press, 1987:50-219
Neergaard, E. B., Andersen, H. C., Hansen, C. C., & Jepsen, O. (1964). Experimental studies on sound transmission in the human ear. III influence of the stapedius and tensor tympani muscles. *Acta Oto-Laryngologica, 57*, 280-286.
Noda, Y., & Pirsig, W. (1974). Anatomical projection of the cochlea to the cochlear nuclei of the guinea pig. *European Archives of Oto-Rhino-Laryngology, 208*, 107-120.
Nolle, C., Todt, I., Seidl, R. O., & Ernst, A. (2004). Pathophysiological changes of the central auditory pathway after blunt trauma of the head. *Journal of Neurotrauma, 21*, 251-258.
Nomura, Y., Harada, T., & Fukaya, T. (1979). Dynamic tympanometry. *Journal of the American Auditory Society, 4*, 190-194.
Mukerji et al. 189
Nuttall, A.L. (1974). Measurements of the guinea-pig middle-ear transfer characteristic. *J. Acoust. Soc. Am. 56*, 1231–1238.
Ochi, K., Ohashi, T., Kinoshita, H. (2002). Acoustic tensor tympani response and vestibular-produced myogenic potential. *Laryngoscope 112*, 2225–2229.
Oliveira CA, Negreiros J, Cavalcante IC, et al. Palatal and middle-ear myoclonus: a cause

for objective tinnitus. *Int Tinnitus J*. 2003;9:37-41.
Oliver DL, Morest DK (1984) The central nucleus of the inferior colliculus in the cat. J Comp Neurol 222:237–264
Oliver, D. L., & Beckius, G. E. (1996). Axons from anteroventral cochlear nucleus (AVCN) that terminate in medial superior olive (MSO) of cat: Observations related to delay lines. *Society for Neuroscience Abstracts, 22*, 127.
Oliver DL, Huerta MF (1992) Inferior and superior colliculi. In: Webster DB, Popper AN, Fay RR (eds) The Mammalian Auditory Pathway: Neuroanatomy. Springer-Verlag, New York, pp 168-221
Oliver DL, Morest DK (1984) The central nucleus of the inferior colliculus in the cat. J Comp Neurol 222:237-264
Ollo, C., & Schwartz, I. R. (1979). The superior olivary complex in C57BL/6 mice. *Journal of Comparative Neurology, 155*, 349-373.
Olsen, W. O., Noffsinger, D., & Kurdziel, S. (1975). Acoustic reflex and reflex decay. Occurrence in patients with cochlear and eighth nerve lesions. *Archives of Otolaryngology, 101*, 622-625.
Osanai, R., Kinoshita, M., & Hirose, K. (2001). Stapedial reflex in myotonic dystrophy type 1 and CTG repeat expansion. *Journal of Neurology, 248*, 1056-1061.
Osen, K. K. (1965). The cochlear nucleus in the common porpoise, Phocaena phocaena. *Journal of Comparative Neurology, 125*, 223-257.
Osen, K. K. (1969). Cytoarchitecture of the cochlear nuclei in the cat. *Journal of Comparative Neurology, 136*, 453-484.
Osen, K. K. (1970). Afferent and efferent connections of three welldefined cell types of the cat cochlear nuclei. In P. Andersen, & J. K. S. Jansen (Eds.), *Excitatory synaptic mechanisms* (pp. 295-300). Oslo, Norway: Universitetsforlaget Oslo.
Ota Y, Oliver DL, Dolan DF (2004). Frequency specific effects of cochlear response during activation of the inferior colliculus in the guinea pig. J Neurophysiol 91: 2185-2193
Otto, SR et al. (2002). "The multichannel auditory brainstem implant update: Performance in 61 patients". *Journal of Neurosurgery* **118** (96): 1063–1071.
Palmer, A. R. (1987). Physiology of the cochlear nerve and cochlear nucleus. *British Medical Bulletin, 43*, 838-855.
Pagano M, Gauvreau K (2000) Principles of biostatistics, 2nd edn. Duxbury, Pacific Grove
Pang, X. D., & Peake, W. T. (1986). How do contractions of the stapedius muscle alter the acoustic properties of the ear? In J. B. Allen, A. Hubbard, S. I. Neely, & A. Tubis (Eds.), *Peripheral auditory mechanisms* (pp. 36-43). New York, NY. Springer-Verlag.
Pickard Ge, Smeraski CA, Tomlinson CC, Banfield BW, Kaufman J, Wilcox CL, Enquist LW, Sollars PJ (2002) Intravitreal injection of the attenuated Pseudorabies virus PRV Bartha in infection of the hamster suprachiasmatic nucleus only by retrograde

transsynaptic transport via autonomic circuits. J Neurosci 22:2701–2710
Pfeiffer, R. R. (1966). Anteroventral cochlear nucleus: Wave forms of extracellularly recorded spike potentials. *Science, 154*, 667-668.
Politzer, A. (1861). Beitrage zur Physiologie des Gehororgans, Vol. 43, Sitzungsberichte der Akademie der Wissenschaften in Wien, Wien.
Politzer A. History of Otology-An English Translation by Milstein S, Portnoff C & Coleman A. Phoenix: Columella Press, 1981;4-69.
Politzer, A. (1981). *History of otology* (S. Milstein, C. Portnoff, & A. Coleman, Trans.). Phoenix, AZ: Columella Press.
Ramirez, L. M., Ballesteros, L. E., & Sandoval, G. P. (2007). Tensor tympani muscle: strange chewing muscle. *Medicina Oral, Patologia Oral y Cirugia Bucal, 12*, 96-100.
Rasmussen, G. L. (1946). The olivary peduncle and other fiber connections of the superior olivary complex. *Journal of Comparative Neurology, 84*, 141-219.
Relkin, E. M., Sterns, A., Schipper, J., Azeredo, W., & Woods, C. I. (2001). The role of the middle-ear reflex in determining the time course of DPOAEs in the rat. *Abstracts of the Association for Research in Otolaryngology, 24*, 6.
Relkin, E.M., Sterns, W., Azeredo, W.J., Prieve, B.A., Woods, C.I., (2005). Physiological mechanisms of onset adaptation and contralateral suppression of DPOAEs in the rat. J. *Assoc. Res. Otolaryngol. 6,* 119–135.
Reuss, S., Al-Butmeh, S., & Riemann, R. (2008). Motoneurons of the stapedius muscle in the guinea pig middle ear: Afferent and efferent transmitters. *Brain Research, 1221*, 59-66.
Reuss, S., Kuhn, I., Windoffer, R., & Riemann, R. (2009). Neurochemistry of identified motoneurons of the tensor tympani muscle in rat middle ear. *Hearing Research, 248*, 69-79.
Rhode, W. S., Smith, P. H., & Oertel, D. (1983). Physiological response properties of cells labeled intracellularly with horseradish peroxidase in cat dorsal cochlear nucleus. *Journal of Comparative Neurology, 213*, 426-447.
Robert H. Margolis LLH. Acoustic Immitance Measurements. In: Ross J. Roeser MV, Holly H. Dunn, eds. Audiology: Diagnosis. New York: Thieme Medical publishing, 2000:382
Roberts KB. Eustachius and his anatomical plates. Newsletter of the Canadian Society for the History of Medicine 1979;April:9-13.
Robertson D (1984). Horseradish peroxidase injection of physiologically characterized afferent
and efferent neurons in the guinea pig spiral ganglion. Hearing Res 15: 113-121.
Roeser, R. J., Valente, M., & Hosford-Dunn, H. (2000). *Audiology diagnosis*. New York, NY. Thieme.
Rood, S. R., & Doyle, W. J. (1978). Morphology of tensor veli palatini, tensor tympani, and dilatator tubae muscles. *Annals of*

Otology, Rhinology, & Laryngology, 87, 202-210.
Rouiller, E. M., Capt, M., Dolivo, M., & De Ribaupierre, F. (1986). Tensor tympani reflex pathways studied with retrograde horseradish peroxidase and transneuronal viral tracing techniques. *Neuroscience Letters, 72*, 247-252.
Rouiller, E.M., Capt, M., Dolivo, M., de Ribaupierre, F. (1986). Tensor tympani reflex pathways studied with retrograde horseradish peroxidase and transneuronal viral tracing techniques. *Neurosci. Lett. 72*, 247–252.
Rouiller, E. M., Capt, M., Dolivo, M., & De Ribaupierre, F. (1989). Neuronal organization of the stapedius reflex pathways in the rat: a retrograde HRP and viral transneuronal tracing study. *Brain Research, 476*, 21-28.
Rouiller, E. M., & Ryugo, D. K. (1984). Intracellular marking of physiologically characterized cells in the ventral cochlear nucleus of the cat. *Journal of Comparative Neurology, 225*, 167-186.
Ryugo DK, Fay RR, Popper AN (2011) Auditory and Vestibular Efferents, vol 38. Springer Science+Business Media, LLC, New York
Saad, M., Dubuc, R., Westberg, K. G., & Lund, J. P. (1999). Anatomical organization of efferent neurons innervating various regions of the rabbit masseter muscle. *Neuroscience, 88*, 927-937.
Saint Marie, R. L., Morest, D. K., & Brandon, C. J. (1989). The form and distribution of GABAergic synapses on the principal cell types of the ventral cochlear nucleus of the cat. *Hearing Research, 42*, 97-112.
Saldana, E., Aparicio, M. A., Fuentes-Santamaria, V., & Berrebi, A. S. (2009). Connections of the superior paraolivary nucleus of the rat: Projections to the inferior colliculus. *Neuroscience, 163*, 372-387.
Salomon, G. (1963). Electromyography of middle ear muscles in man during motor activities. *Acta Neurologica Scandinavica, 39*, 161-168.
Sams, J. M., Jansen, A. S., Mettenleiter, T. C., & Loewy, A. D. (1995). Pseudorabies virus mutants as transneuronal markers. *Brain Research, 687*, 182-190.
Saxon, D. W., & Hopkins, D. A. (2006). Ultrastructure and synaptology of the paratrigeminal nucleus in the rat: primary pharyngeal and laryngeal afferent projections. *Synapse, 59*, 220-234.
Schofield, B. R., & Cant, N. B. (1996a). Projections from the ventral cochlear nucleus to the inferior colliculus and the contralateral cochlear nucleus in guinea pigs. *Hearing Research, 102*, 1-14.
Schofield, B. R., & Cant, N. B. (1996b). Origins and targets of commissural connections between the cochlear nuclei in guinea pigs. *Journal of Comparative Neurology, 375*, 128-146.
Schmued, L.C., Fallon, J.H. (1986). Fluoro-Gold: a new fluorescent retrograde axonal tracer with numerous unique properties. *Brain Res. 377,* 147–154.

Schow, R. L., & Goldbaum, D. E. (1980). Collapsed ear canals in the elderly nursing home population. *Journal of Speech and Hearing Disorders, 45*, 259-267.
Seikel, J. A., King, D. W., & Drumright, D. G. (2000). *Anatomy and physiology for speech, language, and hearing*. San Diego, CA: Singular.
Shannon RV, Fayad J, Moore J, Lo WW, Otto S, Nelson RA and O'Leary M (1993). Auditory brainstem implant: II. Postsurgical issues and performance. *Otolaryngol Head Neck Surg* 108: 634-642, 1993.
Shannon RV, Moore JK, McCreery DB and Portillo F (1997). Threshold-distance measures from electrical stimulation of human brainstem. *IEEE Trans Rehabil Eng* 5: 70-74, 1997.
Shankland, W. E. (2001). The trigeminal nerve. Part IV: The mandibular division. *Cranio, 19*, 153-161.
Shannon, RV et al. (1993). "Auditory brainstem implant. II: Post-surgical issues and performance". *Otolaryngology, Head and Neck Surgery* **108** (108): 635–643.
Shaw, M. D., & Baker, R. (1983). The locations of stapedius and tensor tympani motoneurons in the cat. *Journal of Comparative Neurology, 216*, 10-19.
190 *Trends in Amplification 14(3)*
Shimizu, T., Hayashida, T., Hayashi, H., Kato, S., & Tanabe, H. (1996). Stapedial reflex in amyotrophic lateral sclerosis. *Journal of Neurology, Neurosurgery and Psychiatry, 60*, 544-548.
Shlomo, S., & Silverman, C. A. (1991). Clinical applications of acoustic immittance. In S. Silman (Ed.), *Auditory diagnosis: Principles and applications* (pp. 103-132). Orlando, FL: Academic Press.
Siegel, A., Roeling, T. A., Gregg, T. R., & Kruk, M. R. (1999). Neuropharmacology of brain-stimulation-produced aggression. *Neuroscience & Biobehavioral Reviews, 23*, 359-389.
Smith SB, Parenti A, Macchi V, et al. Hieronymous Fabricius Ab Aquapendente (1533-1619). Clinical Anatomy 2004; 540-3
Smith, J., Sterns, A., Prieve, B. A., & Woods, C. (2005). Anesthetic effects on middle ear muscles and the medial olivocochlear reflex in rats. *Abstracts of the Association for Research in Otolaryngology, 28*, 234.
Smith, M. J., & Brezinova, V. (1991). Stapedius reflex decay test in diagnosis of myasthenia gravis (MG). *Electromyography and Clinical Neurophysiology, 31*, 317-319.
Smith, P. H., Joris, P. X., Carney, L. H., Yin, T. C. (1991). Projections of physiologically characterized globular bushy cell axons from the cochlear nucleus of the cat. *Journal of Comparative Neurology, 304*, 387-407.
Smith, S. B., Macchi, V., & Parenti, A. (2004). Hieronymous Fabricius Ab Aquapendente (1533-1619). *Clinical Anatomy, 17*, 540-543.

Sorra, K. E., Mishra, A., Kirov, S. A., & Harris, K. M. (2006). Dense core vesicles resemble active-zone transport vesicles and are diminished following synaptogenesis in mature hippocampal slices. *Neuroscience, 141*, 2097-2106.
Sotnikov, O. S. (2005). Primary sensory neurons in the central nervous system. *Morfologia, 127*, 75-81.
Spangler, K. M., Henkel, C. K., & Miller, I. J., Jr. (1982). Localization of the motor neurons to the tensor tympani muscle. *Neuroscience Letters, 32*, 23-27.
Spirou, G. A., Brownell, W. E., & Zidanic, M. (1990). Recordings from cat trapezoid body and HRP labeling of globular bushy cell axons. *Journal of Neurophysiology, 63*, 1169-1190.
Stach, B. A., Jerger, J. F., & Jenkins, H. A. (1984). The human acoustic tensor tympani reflex. A case report. *Scandinavian Audiology, 13*, 93-99.
Stevens, S. S., & Davis, H. (1938). *Hearing: Its psychology and physiology*. New York, NY: Wiley.
Strominger, N. L., Silver, S. M., Truscott, T., & Goldstein, J. C. (1981). Horseradish peroxidase identification of the motoneurons to the tensor tympani muscle in rhesus and owl monkeys. *Society for Neuroscience Abstracts, 7*, 54.
Strutz, J. (1981). Efferent innervation of the cochlea. *Annals of Otology, 90*, 158-160.
Strutz J (1982) The origin of efferent innervation vestibular fibers in the guinea pig. Acta Otolaryngol 94:299-305
Strutz J, Bielenberg K (1984) Efferent acoustic neurons within the lateral superior olivary nucleus of the guinea pig. Brain Res 299:174-177
Strutz, J., Munker, G., & Zollner, C. (1988). The motor innervation of the tympanic muscles in the guinea pig. European *Archives of Oto-Rhino-Laryngology, 245*, 108-111.
Terkildsen, K. (1960). Acoustic reflexes of the human musculus tensor tympani. *Acta Oto-Laryngologica Supplementum, 158*, 230-238.
Thompson, A. M., & Thompson, G. C. (1991). Projections from the posteroventral cochlear nucleus to the superior olivary complex in the guinea pig: light and EM observations with the PHA-L method. *Journal of Comparative Neurology, 311*, 495-508.
Thompson AM, Thompson GC (1995) Light microscopic evidence of serotoninergic projections to olivocochlear neurons in the bush baby (*Otolemur garnettii*). Brain Res 695:263-266
Thompson, A. M., Thompson, G. C., & Britton, B. H. (1998). Serotoninergic innervation of stapedial and tensor tympani motoneurons. *Brain Research, 787*, 175-178.
Thomsen, K. (1999). The origin of impedance audiometry. *Acta Oto-Laryngologica, 119*, 163-165.
Topolska, M. M., & Hassmann-Poznanska, E. (2006). Analysis of usefulness of central auditory processes tests in children. *Otolaryngologia*

Polska, 60, 421-424.
Torres-Fernandez, O., Yepes, G.E., Gomez, J.E. (2007). Neuronal dentritic morphology alterations in the cerebral cortex of rabies-infected mice: a Golgi study. *Biomedica 27,* 605–613.
Trueman WH. The dawn of modern dental literature. In: Truman J, Warren GW, eds. The International Dental Journal. Philadelphia: The International Dental Publishing Company, 1898;393-4.
Uchizona, K. (1965). Characteristics of exitatory and inhibitory synapses in the central nervous systems of the cat. *Nature, 207*, 642-643.
Vacher, S. R., Guinan, J. J., Jr., & Kobler, J. B. (1989). Intracellularly labeled stapedius-motoneuron cell bodies in the cat are spatially organized according to their physiologic responses. *Journal of Comparative Neurology, 289*, 401-415.
Van den Berge, H., & Wirtz, P. (1989). Detailed morphology of the tensor tympani muscle of the rat. An integrated light microscopical, morphometrical, histochemical, immunohistochemical and electron microscopical study in relation to function. *Journal of Anatomy, 164*, 215-228.
Van Noort, J. (1969). *The structure and connections of the inferior colliculus. An investigation of the lower auditory system*. Amsterdam, Netherlands: Gorcum.
Venet T, Rumeau C, Campo P, Rieger B, Thomas A, Cour C (2011) Neuronal circuits involved in the middle ear acoustic reflex. *Toxicol Sci.* Jan; 119 (1): 146-55
Verma Ru, Guex AA, Hancock KE, Durakovic N, McKay CM, Slama MC, Brown MC, Lee DJ (2014) Auditory responses to electric and infrared neural stimulation of the rat cochlear nucleus. Hear Res; Apr; 3310:69-75
Vesalius, A. (1725). Anatomicarum Gabrielis Falloppii observationum examen. In A. Vesalii (Ed.), *Opera omnia anatomica & chirugica Cura Hermanni Boerhaave & Bernhardi Siegfried Albini* (Vol. 2). Leiden, Netherlands: Apud Joannem Du Vivie et Joan. & Herm. Verbeek.
Vesalius A. “Anatomicarum Gabrielis Falloppii observationum examen”. In: “Opera Omnia Anatomica & Chirugica Cura Gabriele Fallopio, Gabriele Cuneo, Bernhard Siegfried Albinus, Herman Boerhaave & Prospero Borgarucci”. Lugduni Batavorum, Apud Joannem du Vivie, 1725;770-73.
Vetter DE, Saldana E, Mugnaini E (1993) Input from the inferior colliculus to medial olivocochlear neurons in the rat: A double label study with PHA-L and cholera toxin. Hearing Res 70:173-186
Vetter, D.E., Liberman, M.C., Mann, J., Barhanin, J., Boulter, J., Brown, M.C., Saffiote-Kolman, J., Heinemann, S.F., Elgoyhen, A.B. (1999). Role of a9 nicotinic ACh receptor subunits in the development and function of cochlear efferent innervation. *Neuron 23,* 93–103.
Warr WB (1969) Fiber degeneration following lesions in the posteroventral cochlear nucleus of the cat. Exp Neurol 23:140-155

Warren, E.H., Liberman, MC. (1989). Effects of contralateral sound on auditory-nerve responses I. Contributions of cochlear efferents. *Hear. Res. 37,* 89–104.
Wenthold, R. J. (1987). Evidence for a glycinergic pathway connecting the two cochlear nuclei: An immunohistochemical and retrograde transport study. *Brain Research, 415*, 183-187.
Wells J, Kao C, Konrad P, Milner T, Kim J (2007). Mahadevanen-Jansen M, Jansen ED (2007). Biophysical mechanisms of transient optical stimulation of peripher nerves. Biophys J. 93: 2567-2580

Wersall, R. (1958). The tympanic muscles and their reflexes; physiology and pharmacology with special regard to noise generation by the muscles. *Acta Oto-Laryngologica Supplementum, 139*, 1-112.
Wiener-Vacher, S. R., Guinan, J. J., Jr., Kobler, J. B., & Norris, B. E. (1999). Motoneuron axon distribution in the cat stapedius muscle. *Hearing Research, 133*, 139-148.
Wiley, T. L., & Block, M. G. (1984). Acoustic and nonacoustic reflex patterns in audiologic diagnosis. In S. Silman (Ed.), *The acoustic reflex: Basic principles and clinical applications* (pp. 387-411). New York, NY: Academic Press.
Wilson, R., Shanks, J. E., & Lilly, D. J. (1984). Acoustic-reflex adaptation. In S. Silman (Ed.), *The acoustic reflex: Basic principles and clinical applications* (pp. 329-386). New York, NY: Academic Press.
Windsor, A., Roska, B., Brown, M. C., & Lee, D. J. (2007). Transneuronal analysis of the rat middle ear muscle reflex pathways using pseudorabies virus. *Journal of the Association for Research in Otolaryngology,* Abstract # 605.
Winer JA (1992) The functional architecture of the medial geniculate body and the primary auditory cortex. In: Webster DB, Popper AN, Fay RR (eds) The Mammalian Auditory Pathway: Neuroanatomy. Springer-Verlag, New York, pp 222-409
Woods CI, Azeredo WJ (1999) Noradrenergic and serotonergic projections to the superior olive: potential for modulation of olivocochlear neuron. Brain Res. 836: 9-18.
Wuerthele, S. M., Lovell, K. L., Jones, M. Z., & Moore, K. E. (1978). A histological study of kainic acid-induced lesions in the rat brain. *Brain Research, 149*, 489-497.
Mukerji et al. 191
Ye, Y., Machado, D. G., & Kim, D. O. (2000). Projection of the marginal shell of the anteroventral cochlear nucleus to olivocochlear neurons in the cat. *Journal of Comparative Neurology, 420*, 127-138.
Yoshida,N.,Hequembourg, S.J.,Atencio, C.A., Rosowski, J.J., Libeman, M.C. (2000). Acoustic injury in mice: 129/SvEv is exceptionally resistant to noise-induced hearing loss. *Hear. Res. 141,* 97–106.

Zakrisson, J. E., & Borg, E. (1974). Stapedius reflex and auditory fatigue. *Audiology, 13*, 231-235.
Zakrisson, J. E., Borg, E., Liden, G., & Nilsson, R. (1980). Stapedius reflex in industrial impact noise: Fatigability and role for temporary threshold shift (TTS). *Scandinavian Audiology Supplement, 12*, 326-334.
Zettel, M.L., Zhu, X., O'Neill, W.E., Frisina, R.D. (2007). Age-related decline in Kv3.1b expression in the mouse auditory brainstem correlates with functional deficits in the medial olivocochlear efferent system. J. *Assoc. Res. Otolaryngol. 8,* 280–293
Zipfel TE, Kaza SR, Greene JS. Middle-ear myoclonus. *J Laryngol Otol*. 2000;114:207-209.
Zivic, L., & Zivic, D. (2003). Effect of noise on changes in the acoustic reflex. *Srpski Arhiv za Celokupno Lekarstvo, 131,* 365-369.

14. ERRATA

- I was unable to adapt the endnote computer program to my thesis due to technical reasons. This would have allowed me to list my references with numerical citations.

15. ORIGINAL PUBLICATIONS