The Development of Hearing in Rats; Reliability of wave 1 as a determinant of auditory maturation and contributions of peripheral structure progression

Aminat Saliu

CUNY City College

How does access to this work benefit you? Let us know!

Follow this and additional works at: http://academicworks.cuny.edu/cc_etds_theses

Part of the Biology Commons

Recommended Citation

Saliu, Aminat, "The Development of Hearing in Rats; Reliability of wave 1 as a determinant of auditory maturation and contributions of peripheral structure progression" (2011). CUNY Academic Works.
http://academicworks.cuny.edu/cc_etds_theses/18

This Thesis is brought to you for free and open access by the City College of New York at CUNY Academic Works. It has been accepted for inclusion in Master's Theses by an authorized administrator of CUNY Academic Works. For more information, please contact AcademicWorks@cuny.edu.
THE DEVELOPMENT OF HEARING IN RATS: Reliability of wave 1 as a determinant of auditory maturation and contributions of peripheral structure progression

By
AMINAT SALIU

IN PARTIAL FULFILLMENT FOR THE MASTERS IN BIOLOGY AT THE CITY COLLEGE OF NEW YORK

THE CITY UNIVERSITY OF NEW YORK

MENTOR: Adrián Rodríguez-Contreras
Acknowledgements

Achieving a graduate degree takes hard work, persistence, and prayers. But also important is the requirement of untiring support and guidance of a mentor, family and friends. First, I am sincerely appreciative of my mentor, Dr. Adrián Rodríguez-Contreras, who has reinforced me throughout the task of obtaining this degree with patience and a wealth of knowledge while enabling my independence and personal growth. Thank you for your great leadership and ongoing support within and beyond academia. I could not wish for a better supervisor.

A big thanks to the Louis Stokes Appliance for Minority Participation for the opportunity given to me as a student scientist; I would not have been able to achieve this goal without the scholarship awarded to me. To Dr. Vivek Khatri who has tremendously assisted in conducting this research, thank you for the equipment and setups. I also appreciate the efforts of Dr. Luis Cardoso and Natalia Maldonado for the 3-D micro-CT scans of rat skulls. I would like to thank the reviewers of this manuscript and members of my committee, Dr. Jonathan Levitt whose mastermind I admire and Dr. Karen Hubbard who inspires me as a female scientist.

Next, I will like to extend my deepest appreciation to my family. Thanks to my mother, Sidikat Saliu, whose rhythm of wisdom gets me through hardship and whose uprightness I strive to portray. Thanks to my father and all my sisters for their incessant care and support. Thanks to my fiancé, Mohammed Musah, for his selfless sacrifice and listening ear, not to mention all those days I’ve kept you up explaining my research and practicing my presentations. To all my friends who have always been there for me, I thank you.

Last but not least, I am grateful to God Almighty for keeping and blessing me and those around me. Nothing is possible without Him.
Abstract

Normal auditory development in humans necessitates that structural parts of the ear develop in the first few months of gestation and according to Graven et al (2008) sensory parts of the auditory system develop primarily after 20 weeks of gestational age while the auditory system becomes functional at around 25 weeks of gestation. Unlike in humans where hearing starts in utero, hearing development in rats occurs during postnatal development. As the central nervous system matures, we measure the hearing responses of Wistar rat pups to broadband clicks (1 to 50 kHz) of varying intensities (0 - 82 dB SPL) using the evoked auditory brainstem response (ABR) technique. Rat pups between the ages of postnatal days (PND) 11 and 15 were used for these experiments. Older rats were also used for auditory threshold sensitivity comparison. Auditory responses were not detected on PND11 and PND12 to the highest sound intensity presented and responses could not be reliably evoked at PND13; however, we find that responses rise to a significant value of wave 1 peak amplitude at PND14. The observed rapid change in functional maturity could be due to the maturation of peripheral structures, probably by overcoming a conductive hearing loss. Therefore, clearing of soft tissue from middle ear airway passages could play important roles in determining the hearing onset to airborne sounds in this species. In addition, older rats had higher auditory sensitivity and therefore were able to respond to sounds of lower intensities. In collaboration with Dr. Luis Cardoso’s laboratory here at CCNY, we are able to assess the presence of air in the passages and the degree of ossification in middle ear structures using three-dimensional high-resolution micro-Computer Tomography images from pup skulls. The results show marked differences between PND13 and PND15, suggesting that the magnitude of the hearing response may depend on such structural changes. Altogether, these data suggest that the maturation of peripheral structures play a key role during the onset of hearing.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Introduction</td>
<td>6-12</td>
</tr>
<tr>
<td>Basic physiology of the auditory system</td>
<td></td>
</tr>
<tr>
<td>Embryonic development of the auditory system; importance of the cochlea in auditory signal transduction</td>
<td></td>
</tr>
<tr>
<td>Description of Auditory Brainstem Response (ABR) and use of ABR to assess inner ear function</td>
<td></td>
</tr>
<tr>
<td>Wave 1 amplitude as a measure of auditory maturation</td>
<td></td>
</tr>
<tr>
<td>II. Objectives</td>
<td>12</td>
</tr>
<tr>
<td>To determine how reliable wave 1 is as a measure of auditory maturation</td>
<td></td>
</tr>
<tr>
<td>To examine the extent of structural change in the ear and its influence on auditory maturation</td>
<td></td>
</tr>
<tr>
<td>III. Materials and Procedures</td>
<td>12-14</td>
</tr>
<tr>
<td>Subjects</td>
<td></td>
</tr>
<tr>
<td>Acoustic stimuli, sound system, and calibration</td>
<td></td>
</tr>
<tr>
<td>ABR measurements</td>
<td></td>
</tr>
<tr>
<td>IV. Results</td>
<td>14-18</td>
</tr>
<tr>
<td>Onset of auditory evoked responses; changes in ABR components as a function of age</td>
<td></td>
</tr>
<tr>
<td>Amplitude of response as a function of age</td>
<td></td>
</tr>
<tr>
<td>Differences in threshold as a function of age</td>
<td></td>
</tr>
<tr>
<td>Relation between wave 1 amplitude at threshold and threshold intensity</td>
<td></td>
</tr>
<tr>
<td>Threshold improvement; overnight tracking of auditory response in PND13 animals</td>
<td></td>
</tr>
<tr>
<td>Dependence of auditory function on structural development of rat auditory periphery</td>
<td></td>
</tr>
<tr>
<td>V. Discussion</td>
<td>18-19</td>
</tr>
<tr>
<td>VI. Future research</td>
<td>19-21</td>
</tr>
<tr>
<td>VII. Conclusion</td>
<td>21</td>
</tr>
<tr>
<td>VIII. Reference</td>
<td>22-24</td>
</tr>
<tr>
<td>IX. List of figures</td>
<td>5</td>
</tr>
<tr>
<td>X. Figure legends</td>
<td>32-33</td>
</tr>
</tbody>
</table>
List of figures

Figure 1 ABR response waveform of an adult rat-----------------------25
Figure 2 Increment in wave 1 amplitude during development------------------26
Figure 3 Improvement of auditory sensitivity of animals with age-------------------27
Figure 4 Relationship between response to sound intensities and threshold-----------------28
Figure 5 Response improvements to sound signals with maturation------------------29
Figure 6 Tracking experiment showing threshold improvement with development----------30
Figure 7 Relating structure to functional maturity of rat response-------------------31
1. Introduction

The importance of sensory development is common across all animals and evolutionarily speaking, it is mostly through sensory input that we are able to maintain awareness about our environment. There are many critical aspects of sensory development, one of which is the onset of sensory function. In this work I am interested in addressing this issue in the context of the development of hearing. Therefore I will briefly describe some aspects that are important to understand about how the auditory system functions and what is known about its development.

Basic physiology of the auditory system

The auditory system comprises the ear and neural components in the peripheral and central nervous system. The ear is divided into three parts namely the outer, middle, and inner ear. The outer ear is composed of the pinna (or the auricle) and ear canal; the pinna directs sound (pressure waves) traveling through the air into the ear canal while the ear canal resonates airborne sound and directs it towards the tympanic membrane (or the eardrum) of the middle ear. The middle ear contains the tympanic membrane which transmits sound vibrations from the outer to the middle ear, and a cavity which houses the ossicles that amplify this signal as they transmit it from the middle to the inner ear. The inner ear provides us with the sense of balance and ability to position ourselves in space through the vestibular system. In addition, the inner ear houses the organ of hearing, which enables interaction with our environment and is a significant component of the communication system that is present in many animals, since individuals need to be able to sense sounds made by other members of their own species in order to be able to process and interpret that information (a process that is carried out by the neural components of the auditory system). Hair cells are the sensory receptors of both, the auditory and vestibular systems. Auditory hair cells are located in a sensory epithelium, within the organ of Corti, on a structure called the basilar membrane. On the apical surface of the hair cells are bundles of stereocilia, specialized structures that deflect in response to sound vibration. The vibration sensitivity of the
basilar membrane to sounds of specific characteristic frequency is tonotopically arranged along
the length of the membrane and these vibrations move the hair cell’s stereocilia, generating hair
cell receptor potentials and the excitation of afferent auditory nerve fibers (Robles et al., 2001).

There are two types of hair cells, namely the inner and the outer hair cells. Both inner and outer
hair cells transduce deflections of their sensory hair bundles into electrical signals; however, the
inner hair cells are specifically responsible for transmitting acoustic information, through their
afferent innervation to the brain. The afferent neurons innervating the cochlea synapse onto the
base of the inner hair cells 95% of the time (Shim, 2006). The outer hair cells are responsible for
amplification, frequency selectivity, and tuning of sounds transmitted by the inner hair cells
(Neilsen et al., 2011; Stauffer et al., 2007). The mechanism used by the mammalian outer hair
cells to amplify and tune sounds is effective up to tens of kHz (Neilsen et al., 2011).

*Embryonic development of the auditory system; importance of the cochlea in
auditory signal transduction*

The development of the auditory sensory epithelium within which the organ of Corti and hair cell
receptors are generated is strictly regulated in its proliferation, differentiation, and patterning; any
misregulation can result in auditory dysfunction. Formation of the auditory sensory epithelium
has been enormously studied in the mouse. Transcription factors in the inner ear specify the
auditory sensory domain (Kiernan et al., 2005) while the fibroblast growth factor receptor 1 gene
helps formation of hair cells and supporting cells (Pirvola et al., 2002). Studies have shown that
without transcription factors both hair cells and supporting cells will not develop and markers of
the organ of Corti will not be expressed (Pelling et al., 2005; Pirvola et al., 2002). Hair cells are
known to become post-mitotic in mid-embryogenesis between embryonic days 12 and 15 (Ruben,
1967).
The endocochlear potential is crucial for the normal function of hair cells. The cochlea is a fluid filled organ containing the endolymph and perilymph, which are populated with sodium and potassium ions. The apex of the hair cells is bathed in endolymph, which contains less sodium ions and more potassium ions, while the basal end of the hair cells is bathed in the perilymph, which contains more sodium ions and less potassium ions. The endocochlear potential and the potassium ion concentration are important for the transduction of sound by hair cells. The hair bundles on the surface of hair cells contain stereocilia which are arranged in a stepwise fashion from shortest to longest. The orientation of hair bundle deflection towards the longer stereocilia opens mechanotransducer channels (which are constantly in contact with the endolymph), generates an influx of potassium ions into the stereocilia, and causes depolarization of the hair cells. This influx of potassium is produced mainly by the electrical gradient of the endocochlear potential (Dallos, 1996). Potassium ions are later released from the hair cells into perilymph via basolateral potassium channels (Wangemann, 2002). Although little is known about the mechanism underlying the generation of the endocochlear potential by the stria vascularis, studies have shown that the absence of cells composing the stria vascularis leads to low endocochlear potential and an increase in the threshold of sound pressure levels necessary to evoke depolarization in hair cells (Takeuchi et al., 2000; Cable et al., 1994; Steel and Barkway, 1989).

**Onset of hearing; Description of the Auditory Brainstem Response (ABR) and use of ABR to assess inner ear function**

The Auditory Brainstem Response (ABR) is a set of electrical waves generated by structures of the auditory pathway in response to sounds such as clicks or pure tones. In other words, the ABR is an evoked potential whose components are thought to reflect electrical activity in the auditory nerve and the auditory brainstem. It is known that transmission of sound wave information occurs through the auditory nerve fibers, into the cochlear nucleus (CN), and from the CN signals are transferred into the superior olivary nuclei (SON) of the brainstem, the lateral lemniscus pathway,
and up to the inferior colliculus (IC) in the midbrain. Further activity can also be detected into the medial geniculate nucleus (MGN) of the thalamus, and eventually signals reach the primary auditory cortex (A1). The placement of scalp electrodes determines which components of the transmission chain will be amplified, hence the name ABR.

The ABR technique may be used in humans to assess the level of hearing ability or to check for hearing loss. The ABR may also be used in animals to measure the magnitude of response to acoustic stimuli such as sound presentations of different intensities. Click sounds are mostly used because they are synthetic sounds that contain a broad spectrum of frequencies and are thought to activate responses in large sectors of the cochlea. The ABR measures brainstem electrical signals that originate as a result of action potential firing in auditory neurons. The recorded potential ranges from nanoVolts to microVolts and is plotted against time in milliseconds. Obtaining responses at different sound intensities enables the measurement of hearing sensitivity which is determined by the minimum intensity of sound that evokes a reliable response above baseline. Each factor of auditory development previously explained is important for sound transmission and thus, auditory function both in animals and in humans.

Studies in humans

Research has shown that hearing in humans begins in utero (Sininger et al., 1997), and continues maturing through puberty and into adulthood. ABR measurements have been used to study hearing sensitivity in infants and have shown that newborns are less sensitive to sound stimulation than adults. In 1979, researchers found that click sound ABR thresholds in newborns were between 10-20dB higher than adult thresholds. This result is in line with the results of other studies such as that conducted by Sininger et al. (1997). While the ABR is not influenced by level of attentiveness or arousal (Starr, 1976), it has been determined that auditory thresholds of newborns tend to be lower using the ABR method than when using other techniques such as behavioral audiometry (Kaga and Tanaka, 1980). Since the ABRs are conducted in an
environment that produces minimum background noise than behavioral methods, it is safe to say the ABR gives better result of the state of peripheral auditory system maturation in newborns.

Studies in animals

Similarly, the ABR measurement has been used to study auditory development in several animals such as rats, mice, gerbils, cats, ferrets, and members of the kangaroo family known as the tammar wallaby. All these results combined enlighten us that development across mammals has striking similarities, despite species-specific differences in developmental timeline. McFadden et al, 1996 investigated the commencement of auditory function and maturation through threshold sensitivity in the Mongolian gerbil. Consistent with previous research done, this group showed that auditory evoked responses to sound stimuli are elicited only when structures of the ear including the cochlear and middle ear bones are developed enough to conduct mechanical vibrations; this was in gerbils as young as PND 10 (in only two animals), to intensities as high as 90-110 dB. Reliable ABRs to click stimulation in gerbils experimented on were not obtained until PND16 (McFadden et al, 1996).

Wave 1 amplitude as a measure of auditory maturation

Many previous studies conducted to investigate the start and maturation of auditory function focused on using threshold of sound intensity as a gauge to measure improvements of responses to sound. Example of such studies are those done by Zhou et al (2006) who assessed hearing sensitivity in ten different strains of adult mice, McFadden et al (1995) who examined auditory sensitivity in Mongolian gerbils and Sininger et al (1996) who compared ABR thresholds of infants and adult humans.

Wave 1 is the first clear wave within an ABR waveform with latency between 2 and 3.5 ms of sound presentation and according to Möller et al (1983) wave 1 represents activity in the auditory nerve. Based on this knowledge and also knowing that the auditory nerve fibers carry sound
information from the cochlea to the brain, wave 1 should be a good determinant of auditory function development and maturation. Supporting this reasoning is a study done by Ngan and May reported in 2001. In this study the authors looked at the relationship between thresholds obtained with ABR and those obtained directly from auditory nerve recordings in normal cats. They showed that in normal cats ABR threshold was about 25dB higher than auditory nerve fiber threshold. Although the focus of their research was different, their data support the fact that measuring activity of the auditory nerve fiber, rather than measuring ABR threshold, is a good indicator of hearing improvement in rats. Kujawa and Liberman recently showed that wave 1 can be used as a quantitative measure of hearing function in adults (Kujawa and Liberman, 2009). To the best of my knowledge no one has tried to use wave 1 amplitude to assess the emergence of hearing function during development. In this study I hypothesized that as development progresses the magnitude of hearing response, as signified through wave1 measurement will increase.

In this study we show that wave 1 is an accurate index of hearing ability in rats, that formation of the ossicles within the middle ear in addition to soft tissue clearance of the middle and outer ear are important milestones of auditory development, and that just as in human individual rat development varies depending on their physiological makeup. In addition, to confirm what was shown in other studies, thresholds were measured to evaluate development of the auditory periphery. Furthermore, we show that opening of the ear canal must take place to achieve auditory functions as there was no animal without an open ear canal that gave auditory evoked responses; however, an open ear canal does not guarantee hearing abilities as there were three postnatal day 15 animals that had open ear canals but could not give auditory evoked responses to moderately loud sounds. The timeline of rat auditory brainstem response was determined in addition to developmental changes in the magnitude of hearing responses of rat pups and the threshold differences among rats of different ages. In collaboration with Natalia Maldonado, a graduate student in Dr. Luis Cardoso’s Biomedical Engineering laboratory here at CCNY, we were able to map out the structural changes in the outer and middle ear that rat pups undergo.
during hearing development and determine the dependence of hearing maturation on structural development.

**Materials and Procedures**

2.1 Subjects

All experimental procedures regarding the use of animals were reviewed and approved by the Institutional Animal Care and Use Committee at City College of New York. All rats (Wistar strain; obtained from Jackson Labs) were reared in a standard rat cage at the City College animal facility where food, drink, room temperature, and dark/light cycles were monitored until the day of experiment. Experiments were performed one rat at a time. Ages used were between PND11 to PND15 (with one day intervals), and PND21 and PND29; irrespective of gender all animals were handled similarly. The first 24h following birth is PND0. Animals were chosen at random from the litter for measurements. All experimental animals appeared to be in good health at the time of experiment judging from physical appearance, physical activity, and weight of the animal. A total of 49 animals were used from 28 litters; PND11 (n= 4), PND12, (n=6 ), PND13 (n= 11), PND14 (n= 13), PND15 (n= 12), and PND21 (n=3).

2.2. Acoustic stimuli, sound system, and calibration

Click sound auditory stimuli were produced with Tucker-Davis Technologies (TDT) System hardware using the SigGen signal generator, amplified by a Medusa preamplifier and delivered through a free-field Kanetec MB-FX speaker placed 34 cm horizontally from the right ear. The ABR stimuli were 20 µsec long broadband (1-50 kHz) clicks presented at a rate of 40 Hz with intensities varying from 0dB to 82dB sound pressure level (SPL).
2.3 ABR measurements

ABR is an electrical signal recorded from the brainstem after the presentation of a sound. Wistar rats are first briefly anesthetized with a mixture of oxygen and isoflurane for the purpose of weighting. The rat body weight is factored into the initial dose of ketamine anesthesia (41.7mg/kg) that will be administered during the course of the surgical procedure. Xylazine is also administered alongside ketamine, at a concentration of 2.3mg/kg (drug concentrations may be further diluted depending on the rat’s physiological condition, evaluated by toe pinch reflex). These drugs were administered intraperitoneally. Supplemental doses of both drugs were administered as needed throughout the length of the recording to maintain a deep level of anesthesia. The anesthetized animal was placed on a thermostatically controlled heating pad with body temperature maintained at 37 degree Celsius.

A study done by Ping et al in 2007 showed that transverse mastoid needle electrode placement during ABR recordings in rats reflects a measure of neural activity in the auditory nerve. Therefore electrodes (model GRASS RA4LI) that register electrical signals from the head are inserted through the scalp into the muscles at the back of the ears (the mastoid); the active electrode is inserted into the back of the ear where activity is being recorded (usually the right ear), the reference electrode is inserted into the back of the opposite ear, and the ground electrode is inserted into the back of the neck, immediately below the skin. Sound of varying intensity was presented to the rat pup in a sound-attenuating chamber; intensity is presented in decrements of 5 dB SPL ranging from 82dB SPL to 0dB SPL using a built in attenuator. Clicks of different intensity were repeated in three sets of trials, with a thousand presentations in each trial.

Scalp electrodes transmit brain response signals that are amplified with the Medusa preamplifier, signals are digitized through an A/D board and shown as waveforms on a connected computer screen. ABR measurements generate waveforms as an indication of the magnitude of hearing response to varying sound intensities. Representative waveforms of auditory response are saved
and inspected at the end of sound presentation and analyzed more carefully offline. Graphs were generated using Igor software (Wavemetrics).

3. Results

3.1. Onset of auditory evoked responses; changes in ABR components as a function of age

3.1.1. Amplitude of response as a function of age

In the ABR measurements we focused on wave 1 (Figure 1), a reflection of activity in the rodent auditory nerve (Moller, 1983), whose fibers carry sound information from the cochlea to the brainstem. The magnitude of wave 1 response induced by click sound stimulation was measured as the positive rise phase from response onset to the peak of the wave. As shown for a recording obtained from an adult animal (Figure 1) the amplitude of wave 1 is small, of only a few microvolts. Therefore it is important to note that performing similar experiments in rat pups presents a challenge, since they have small head size and immature nervous systems.

Although sound of various intensities were presented to each rat, an arbitrary moderately loud intensity of 57 dB SPL was chosen to probe hearing in different animals. Animals of different ages were tested throughout the course of this study, ranging between PND11 to PND15. The Y-axis in figure 2 shows wave amplitude in nanoVolts while the X-axis shows the time scale of wave emergence (latency, in milliseconds; Figure 2A-E) and the age of animals in days (Figure 2F). The click stimuli at 57 dB SPL up to 82 dB SPL presented to PND11 and PND12 rats elicited no clearly evoked wave 1 response (Figure 2A,B); criteria for defining wave 1 is having a clearly definite waveform with a latency between 2 and 3.5ms. The latency is defined as the time it takes to elicit a wave 1 response and is measured from the time of sound presentation to the peak emergence of the first wave. Similarly, at PND13 (Figure 2C) none of the animals produced a reliable wave 1 response to all the intensities played except one which responded at the highest
intensity of 82 dB SPL (data not shown). Animals of these age groups are considered to have very high thresholds and could probably elicit responses to higher sound intensities, higher than our click sound intensity generating equipment permits. So far, these measurements show that hearing to 57 dB SPL sound intensity can be reliably tested beginning at PND14 (Figure 2D) and most animals respond and continue to mature at PND15 (Figure 2E). Figure 2F shows the increase in wave 1 magnitude as a function of age; as animals mature, activity of the auditory nerve fibers increase as indicated by changes in wave 1 amplitude (PND11, n= 4; PND12, n= 6; PND13, n= 11; PND14, n= 13; PND15, n= 12). Despite responses obtained at PND14 and PND15, the amplitude of these responses are still very immature when compared to the adult wave 1 amplitude shown in figure 1. Variations in individual traces in PND11, PND12, and PND13 animals explains that auditory signals are not reproducible and thus confirms that these traces cannot be considered as wave 1 responses. From this result the hypothesis that as development progresses the magnitude of hearing response, and wave1 measurement increase is supported. This graph shows both the three sets of trials during the sound presentation in grey and the average for all the trials in black; for simplicity and to explain my results clearly and efficiently, I use the average of the trials for subsequent figures.

3.1.2. Differences in threshold as a function of age

To confirm what has been shown in previous studies, auditory sensitivity was assessed through thresholds. Threshold is defined as the intensity at which an animal responds to at least two out of all three sound presentation repetitions; this response must have at least one clearly defined wave in the waveform and cannot be reproducible at any lower intensity. Stimuli with intensities below the threshold intensity are considered not detectable. The presence of a response is defined through visual examination. Thresholds of PND11, PND12, and PND13 are assumed to be really high and the limitations of our sound equipment do not enable us to present sound intensities higher than 82 dB SPL; therefore, there is no reported data regarding threshold for these age
groups. Not shown here is the exception of one PND13 animal that elicited a reproducible response to an 82 dB SPL sound intensity.

In figure 3, I show using three animals at different ages of PND14, PND15, and PND21 that sensitivity increases with age, causing a reduction in threshold intensity level; with the youngest animal having the lowest sensitivity and vice versa. As shown in this figure, thresholds improved to from 57 dB in PND14, to 42 dB in PND15 and to about 37 dB in PND21. Furthermore, the relation between the click sound stimulus and hearing sensitivity of animals was studied with respect to the animal’s age. Figure 4A shows wave1 amplitude as a function of intensity; this figure simply reiterates that as intensity increases wave1 amplitude increases and this improves as a function of age. In figure 4B and 4C (PND14, n= 7; PND15, n= 4; PND21, n= 3) threshold intensity is plotted as a function of age; the sound intensity to which PND21 animals respond is lower than that to which PND15 animals respond, which in turn is lower than that to which PND14 animals respond. This specifies that auditory sensitivity is lowest and threshold intensity level is highest in the PND14 rat pup; as predicted, younger animals have a higher threshold while the threshold of older animals significantly decreased.

3.1.3. Relationship between auditory response magnitude and threshold of auditory stimulus

Due to independent maturation rate of each animal, responses to auditory stimulation varied in magnitude. It was therefore determined if a low response to the intensities of sound stimulus correlated with an increased threshold, or if threshold intensity remained approximately equal (but lower in magnitude) to other animals of higher response to the same sound stimulus. Based on analysis of current data from each of PND14, PND15, and PND21 age groups, acoustic threshold of animals with lower acoustic response seems to be higher while the threshold of animals with higher acoustic response are lower. This suggests a correlation between low response to the intensity of sound stimulus and increase in threshold. Comparing across age
groups, for most younger animals, even at the increased threshold the value remains lower than threshold values for higher response older animals (Figure 5; PND14, n= 7; PND15, n= 4; PND21, n= 3).

3.1.4. Threshold improvement; overnight tracking of auditory response in PND13 animals

While other studies have indicated that sensitivity to airborne sounds in rats begins between PND12 and PND14 (Geal-Dor et al, 1993) and in gerbils between PND10 and PND12 (McFadden et al, 1996), we show that although wave 1 can be induced in some PND13 rats using intensities as high as 82 dB SPL (for only one rat, data not shown) under our standard conditions a reliable wave 1 response to 57 dB SPL begins at PND14. This was proven to be true from the auditory response tracking experiment that was performed; the ABRs of two rats were measured starting from PND13 and ending at PND14 at intervals of six hours. We confirmed from the measurements that both rats did not elicit a response at PND13 but on PND14 one of the rats did; a firsthand experience proving that maturation of hearing in Wistar rats is reliably tested starting from around PND14, under our experimental conditions (Figure 6).

3.1.5 Dependence of auditory function on structural development of rat auditory periphery; Comparison of rat auditory response and morphology of outer and middle ear

We are also interested in mapping out patterns of structural development in the outer and middle ear of rat pups, in order to search for a relationship between structures of the ear and function that the ear performs. We observe increased magnitude of hearing responses between PND11 and PND15 rat pups and traced underlying changes in the outer and middle ear structures leading to these differences observed. The extent to which form is correlated with function was examined. Using a three-dimensional high-resolution micro-Computer Tomography to scan the skull of
these rats, Natalia Maldonado, a graduate student in Dr. Luis Cardoso’s Biomedical Engineering laboratory, obtained images that can be used for analysis of structural variations. It was identified that soft tissue clearance occurs from inside and extends outward; soft tissue clears from the middle ear cavity first before that of the auditory canal. Comparing PND13 through PND15 rats, it is known that a PND13 rat does not give hearing response at 57 dB SPL and its ear structures should look different from that of a PND15 rat, which is well developed and gives good hearing response. Images obtained from these two rats support our hypothesis (see Figure 7). Despite results of other studies stating that opening of the auditory canal is a less important factor compared to tissue clearance and ossification (Geal-dor et al., 1993) we observe that although opening of the auditory canal occurs secondary to soft tissue clearance and ossification, the extent of this opening, is a good indication of the magnitude of rat hearing response to sound. However it is clear that functional development of hearing is not only due to conductive or structural factors; it also depends on other factors such as sensorineural components of maturation, and these components may be underdeveloped in younger rats. In PND15 rats, conductive hearing is fully developed while sensory hearing continues to mature.

4. Discussion

The complexity of brain development is common to both vertebrates and invertebrates; bringing about different timelines of development in specific species. Common to all animals, the brain development begins with the induction of epidermal ectoderm by the mesoderm/notochord. This development continues through several stages, is maintained through puberty, and lengthens into adulthood; however, in its complexity, the timing for which the brain constituents develop varies among animals while the sequence remains astonishingly preserved in mammals. For example, rats have a gestational period of about 22 days and 165 days in macaque monkeys but in these two mammals, the retinal ganglion cells, are generated before amacrine cells, retinal projections reach the optic chiasm long before they separate into ipsilateral and contralateral patterns in the lateral geniculate nucleus (Clancy et al., 2008). This appears to be true also in humans, which
have a gestational period of between 37 and 42 weeks (Norwitz, 2007). Although the sequence of development is conserved, the timeline of development is different.

We show under our experimental conditions, through an auditory response tracking experiment, that a reliable wave 1 response to 57 dB SPL in Wistar rats begins at PND14. The fact that out of the two rats used for the tracking experiment, only one elicited a response at PND14 also explains further that each individual rat growth is physiologically different. This result is supported with reports by Smith and Draus (1987) who showed that using click sound stimuli on gerbils, they did not start to obtain reliable ABR response until PND14, the earliest age where ABRs obtained were replicable. These studies by Geal-Dor et al and McFadden et al being able to elicit responses in rodents as young as PND10-12 indicates that opening of the auditory canal is not necessary for the onset of auditory function since it is known that at these ages the auditory canal is still closed. At PND15 almost all the animals evoked wave 1 response. Although the auditory canals of animals in this age group are open, the small proportion (25%) of animals that did not elicite a wave 1 response show that even at this age group some animals have really high threshold levels due to variations in physiological development. Note that the inability of an animal to respond to the sound intensity presented does not mean the animal is deaf, it may mean that the threshold intensity of the animal is beyond what was presented to it.

5. Future research

Common to previous studies and confirmed by our studies, the auditory brainstem response increases as animals develop (Blatchley et al, 1987). This is seen through the well-defined waveforms. Ultimately, we would like to show the kind of changes that occur in animals reared under enriched conditions. It is widely known that environmental factors also influence the development of sensory organs such as the brain. For example, rearing rats in the dark decreases the vascular density and thickness of the visual cortex (Lafuente et al, 1996) while rearing rats in an enriched environment increases cortical thickness (Diamond et al, 2001) and neurogenesis in
the hippocampus (Kim et al., 2006), exposing young rats to sound speeds up reflex emergence and development (So et al., 2009), and a report by Engineer (2004) shows that environmental enrichment results in rapid remodeling of rat cortical responses in less than two weeks and auditory recordings made under anesthesia indicate that enrichment not only increases the number of neurons activated by any sound, but also make the primary auditory cortex neurons more sensitive to quiet sounds, more selective for tone frequency, and altered their response latencies. This study did not explicitly show, however, if these animals had significantly lower threshold than animals reared in a standard environment.

As the auditory cortex is important in development of the auditory system and is easily influenced by environmental factors (Graven et al., 2008), behavioral and neural differences between animals reared in a standard environment and animals reared in an enriched environment could be quantified; measuring thresholds at PND15 will dependably enable us to quantify the number of animals with either a high threshold, a low threshold, or no response to the presented click stimuli. In line with results that have been obtained in previous environmental enrichment studies as discussed above, I predict that in an environment where animals are enabled to extensively explore their environment using a variety of available toys and tools, physical interaction (such as licking and grooming) between the dam and pup will increase which may lead to acceleration of the pup development. All PND15 animals should elicit responses to the moderately loud click sound of 57 dB SPL presented. In addition, it could be determined if hearing responses to 57 dB click stimuli will begin prior to PND14 in environmentally enriched animals.

Changes in structural development of the middle and inner ear could also be determined. Previous research that have looked into the effect of environmental enrichment states that rats raised in this setting show improved cognition, enhanced neural plasticity and speedy visual system development (Cancedda et al., 2004). Therefore, there must be factors stimulating cell growth and neurological maturation accounting for the changes observed. Specifically, it has been shown that
enriched environment affects the expression of genes in the cerebral cortex and the hippocampus that determine neuronal structure (Berchtold et al., 2002). At the molecular level, this occurs through increased concentrations of the neurotrophins and changes in brain-derived neutrophic factor (BDNF). BDNF acts on certain neurons of the central nervous system and the peripheral nervous system, helping to support the survival of existing neurons, and encourage the growth and differentiation of new neurons and synapses. In the brain, it is active in the hippocampus, cortex, and basal forebrain—areas vital to learning, memory, and higher thinking. A longer term aim of this research is to identify some of those signaling factors that play an important role in the maturation of the auditory periphery. Identifying differential expression of certain proteins in both control and experimental rats will take us a step further towards classifying the proteins that play a key role in the maturation of auditory periphery. Understanding auditory development and the factors contributing to hearing will enable us to find preventive measurements for congenital auditory malfunctions and therapies for hearing loss in both children and adults.

6. Conclusion

Auditory brainstem response measurements as used in this study enabled us to establish that wave 1 serves as a good index of peripheral maturation, along with threshold sensitivity. As illustrated, wave 1 amplitude increases with age while sensitivity to sound intensities also improves and thus decreasing threshold intensity level. Age related increase in auditory response amplitudes characterize the effect of overcoming conductive hearing loss by soft tissue clearance of auditory peripheral structures and opening of the auditory canal as maturation progresses.
References


Figure 1
Figure 3

PND 14

- 57dB
- 52dB
- 42dB
- 37dB
- 32dB

PND 15

- 57dB
- 52dB
- 47dB
- 42dB
- 37dB
- 32dB

PND 21

- 57dB
- 52dB
- 47dB
- 42dB
- 37dB
- 32dB
Figure 4

A

B

C

Figure 4
Figure 5

- **PND 14**

- **PND 15**

- **PND 21**
Figure 6

A

B
Figure 7

P13

P15

Ossicle
Auditory canal
Middle ear cavity
Figure Legends

Figure 1. Example of a representative ABR waveform from an adult rat. The amplitude of wave 1 is indicated in a. The peak of wave 1 (arrow) is indicated in b. Wave 1 occurs at a latency of about 2.5ms, shown in c. Note the amplitude of wave 1 is in the microvolt range.

Figure 2. Increment in wave 1 magnitude during development. (A, B, C) Waveforms from PND 11, PND12, and PND 13 rat pups; As seen there are no clear responses and no wave 1 is observed. (D) At PND 14 a reliable measure of wave 1 can be measured, but is still smaller than the wave 1 recorded from a PND 15 rat pup, (E). (F) Graph showing responses across ages, wave 1 average for each animal was used to plot this graph(PND11, n= 4; PND12, n= 6; PND13, n= 11; PND14, n= 13; PND15, n= 12). For all rat pups, the sound intensity is at 57dB SPL. Note that the amplitude of wave 1 is in the nanoVolt range, however, latency and reliability across three trials indicates a similarity with the evoked potential recorded in the adult rat in Figure 1.

Figure 3. Auditory sensitivity of animals improve with age. (A) PND14 animals show the highest threshold intensity at 57dB, the threshold intensity of PND15 is intermediate at 42dB (B), while the threshold of PND21 is lowest at 37 dB (C). Arrow indicates the line that intersects all wave 1s. Wave 1 latency is between 2 ms and 3.5ms.

Figure 4. Response improvement to sound signals with maturation. (A) Wave 1 amplitude increases as sound intensity increases, with a greater signal response in PND21. (B, C; PND14, n= 7; PND15, n= 4; PND21, n= 3) Thresholds of older animals
are lower than those of younger animals; the sound intensity at which older animals respond in at least two out of three sound presentations is lower than the sound intensity at which younger animals respond in at least two out of three sound presentations. Overlapping threshold levels were offset by a point to enable visualization of all points.

**Figure 5.** Determination of relationship between response to the intensities of sound stimulus and threshold within each age group. Within each age group (PND14, n= 7; PND15, n= 4; PND21, n= 3), results show that animals with lower response have higher thresholds while the threshold of animals with higher response are lower, suggesting a correlation between low response to the intensity of sound stimulus and increase in threshold. This correlation seems to be more apparent within PND14 age group having seven individual points.

**Figure 6.** Tracking experiment from PND13 to PND14. (A) ABR measurements on PND13 shows no measurable response of wave 1. (B) By PND14, this animal was able to respond to 82dB SPL.

**Figure 7.** In PND13 rat only the bulla is cleared of soft tissue (air filled black regions), while the auditory canal is still blocked. Also, the ossicles are not as developed as in the PND15 rat. This rat experiences conductive hearing loss. In contrast, both the bulla and auditory canal of PND15 rat is cleared of soft tissue. The ossicles of this rat are also fully developed. This rat does not experience conductive hearing loss and has a clear response to sound. Blue trace is cut in PND13 recording.