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# Normal and Mutant Murine auditory brainstem responses (ABRs)

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Normal and Mutant Murine Auditory Brainstem Responses (ABRs)

Kristin Marie Shearer

A dissertation submitted to the Graduate Faculty of

JAMES MADISON UNIVERSITY

In

Partial Fulfillment of the Requirements

for the degree of

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## Abstract

Spatial organization and topographic maps begin in the periphery and are preserved throughout the central auditory system. The Eph and ephrin family of signaling proteins is involved in the patterning and arrangement of auditory afferents which code information about frequency, intensity, and time. The present study utilizes the ABR as a physiological measure of the electrical potentials occurring in the brainstem following acoustic stimulation. We recorded this measure following click and tone pip stimulation in ephrin-B3 and EphA4 deficient mice at 2-3 months of age and compared them to wild type controls. Significant findings included elevated auditory thresholds, prolonged latency to waveform peaks, and diminished peak amplitudes among the mutant groups. The physiological differences between the wild type and mutant groups provide strong evidence of the Eph/ephrins in establishing a fully functional auditory system.

## **Introduction**

The ability to localize sounds and to extract meaningful information from a background of noise requires information about the frequency, intensity, and timing of sound stimuli. These complex processes require precise organization throughout the afferent auditory system, which begins at the cochlea and continues in the central nervous system from the auditory brainstem through the midbrain to diencephalic and telencephalic structures. Functionality of this system relies on molecular mechanisms to align and to shape these connections. The family of Eph receptors and their ligands, the ephrins, is known to be important in the early patterning of auditory afferents. Recent anatomical research has investigated the roles of Eph/ephrins in many converging inputs to the auditory midbrain. The inferior colliculus (IC) is the prominent auditory nucleus in the midbrain. In mice, the IC consists of a larger number of inhibitory, rather than excitatory, receptor fields. Inhibition along the auditory pathway is important for processing of timing and intensity cues which contribute to binaural processing when both ears are working together. The purpose of the present study is to study simple functions of time and intensity in the early auditory evoked responses, up to the level of the IC. If a loss of functioning Eph receptors detrimentally affects the development of precise connections, this influence is expected to show up in evoked potentials that are abnormal in either latency (time) or amplitude (intensity) or both (latency-intensity functions). This thesis will examine a set of 5 peaks, generated at peripheral and central auditory nuclei up to the IC.

Auditory brainstem responses (ABR), and evoked potentials in general, reveal detailed information regarding the synchronicity of auditory activity. Latencies of various peaks are affected by frequency and intensity. Thus a relatively complete ‘picture’ of information about the frequency, intensity, and timing of auditory processing



as it proceeds up the ascending pathway can be obtained from ABRs. Well-formed structure of various peaks in the evoked potentials reveals functional circuitry that has presumably organized appropriately.

Peaks at various latencies reveal precision of organization at the level of the auditory brainstem and midbrain. Therefore, the independent variables in this study are frequency and intensity of stimuli and the mouse genotype. The principle dependent variable in this study is the ABR threshold, defined as the average between the lowest intensity at which reliable waves can be recorded and the first intensity the response was absent at various frequencies. The latencies, amplitudes and general structure of various well known peaks in the evoked potential are also presented to show activity of brainstem relay nuclei as the auditory signals ascend the afferent pathway. Four groups of mice are studied: two with mutation in ephrins, one without Eph receptors (a true knockout), and a control group (termed wild type).

## **Background**

### **A review of molecular biology: Ligands and receptors.**

To begin this study, a basic understanding of biological processes is important. Ligands are proteins which bind to receptors. Receptors are special proteins on the surface of cells that are capable of receiving stimulation and causing some form of intracellular response. Tyrosine kinases are a family of receptors responsible for controlling the development of neurons. Any mutation to the biological function of these receptors can “result in unrestrained proliferation and transformation,” thus disrupting typical developmental and regenerative procedures (Cadena & Gill, 1992, p. 2332). Receptor-ligand binding results in increased kinase activity. Dimers are formed, which are “physical interactions between related proteins,” and the receptors are internalized

from the cell surface in order to reduce signaling (Klemm, Schreiber, & Crabtree, 1998, p. 569).

*Eph receptors* and *ephrins* are required for ordering of the auditory midbrain connections and have various functions in development. At least 14 members have been identified in vertebrates (Flanagan & Vanderhaeghen, 1998). While variation exists between expression in specific tissues of Eph receptors or ephrins across species, there is an overlap of function and expression which, when activated, will result in the same type of cellular response. Therefore, the Eph family involvement is meant for “complex developmental regulation rather than to control distinct cell responses” (Wilkinson, 2001, p. 156).

Eph-ephrin interactions can be bidirectional (ephrin-to-Eph, *forward*; Eph-to-ephrin, *reverse*) and are divided into two subclasses (A and B). The two classes differ in membrane anchors; class A through a glycosyl-phosphatidylinositol (GPI) linkage and class B by transmembrane proteins (Holder & Klein, 1999). Ephrin-A ligands generally bind with EphA receptors, and ephrin-B ligands generally bind to EphB receptors. However, in the case of ephrin-B2 and ephrin-B3, the B ligands also activate EphA4 and allow bidirectional signaling (Pickles, Claxton, & Van Heumen, 2002). Forward and/or reverse signaling can be seen as well as increased or decreased outgrowth of axons (Cramer, 2005). It has been shown that, “given their bidirectional effects, B class ligands and receptors, or B class ligands with EphA4, are necessary if cell segregation is to be set up or maintained by means of the Eph system” (Pickles et al., 2002, p. 208).

The Eph receptors and ephrins are important for activity-independent processes to occur in development. These molecular markers direct axons to the appropriate target and allow cell-to-cell interaction for the development of complex networks (Gabriele et al., 2011; Gabriele, Shahmoradian, French, Henkel, & McHaffie, 2007). Continuous

topographic maps are developed when axons connect to the proper target cells to generate a rough map which is further refined by positional labels. The Eph family has “complementary graded expression patterns of a receptor-ligand pair” that result in formation of topographic maps (Tessier-Lavigne, 1995, p. 347). In the auditory system, spatial organization and topographic maps begin in the periphery and continue to higher central auditory levels.

The Eph proteins have several roles in normal hearing and in the development of the central auditory structures. Within the cochlea, they are involved in structural development and regulation of ion concentration levels. Evidence exists of selective targeting of cochlear innervation within the auditory system. Spiral ganglion cells have neuronal connections expressing ephrin-A2 and EphB1 with some expression of ephrin-B1 and ephrin-B2 (Pickles et al., 2002). Eph-ephrin interactions are also involved in the development of tonotopic order and connectivity at the level of the cochlear nucleus and superior olivary complex (Miko, Nakamura, Henkemeyer, & Cramer, 2007). The efferent system which originates in the brainstem also has been shown to depend on proper functioning of the Eph proteins and topographic mapping in the central nervous system.

Before the onset of hearing, multiple layered inputs converging at the IC create defined topographic maps. The IC is organized such that high frequencies are coded in the ventromedial part of the central nucleus and low frequencies are coded in the dorsolateral region, with high frequency regions maturing earlier than low frequency regions (Kelly, Liscum, Van Adel, & Ito, 1998). Some of the mapping that occurs is intrinsic to the auditory system, but some is dependent on activity (Gabriele et al., 2011). The layers within the IC are made up of combinations of various inputs, including those arising from the cochlear nucleus, superior olivary complex, and nuclei of the lateral lemniscus. Having overlapping and non-overlapping patterns in the central nucleus of the

IC serves to “determine the innervation and organizational scheme for functional compartments of neurons” and allows for the processing of different auditory tasks (Gabriele et al., 2007, p. 69). The precise arrangement of these pathways begins prior to the onset of hearing and refines in the early stages of development in order to create a functional auditory system.

### **General Statement of the Problem**

The purpose of the present study is to determine functional consequences in Eph-ephrin mutant mice utilizing physiological measures. The fact that the genes found in the mouse are essentially the same as the genes found in humans makes it a useful animal model for research (Spencer & Kumar, 2002). It is well established that Eph-ephrin interactions play a pivotal role in the development and organization of the auditory system, yet few studies have assessed their importance from a physiological or behavioral perspective. Previous studies have focused mainly on the development within the cochlea and spiral ganglia, while less is known about the role of the Eph-ephrins in the central auditory system. Electrophysiological studies of mutant mice have analyzed amplitudes of the peripheral waves I-III in the auditory brainstem response (ABR); however, the present study focuses on wave V which reflects activity in the IC. To complete the study, threshold ABRs and neurodiagnostic ABRs will be obtained in normal and mutant adult mice at 2-3 months of age.

### **Literature Review**

The mouse cochlea reaches maturity at 8 days old and consists of two turns. The cochlear potentials reach adult levels of sensitivity by 14 days old (Mikaelian & Ruben, 1965). The frequency limits of hearing in a mouse range from 500 Hz to 100 kHz with optimal hearing sensitivity at 15 kHz (Ehret, 1974). Behavioral audiograms revealed optimal sensitivity between 8 kHz and 24 kHz with decreased thresholds in the

frequencies above and below (Radziwon et al., 2009). The control strain used in this study (C57BL/6J mice) were found to have early onset hearing loss beginning in the high frequencies at 8 months of age, therefore measures were taken to ensure that testing was completed well before these processes began (Parham, 1997).

The ABR is a popular method for assessing electrical potentials in the mouse auditory system following acoustic stimulation (Parham, Sun, & Kim, 2001). ABRs are a short- latency auditory evoked potential which can help in determining site of lesion and estimation of hearing sensitivity in subjects with healthy middle ear function. Mouse ABRs are closely related to those in cats and humans, which makes mice a useful animal model of the auditory pathways (Henry, 1979). The mouse ABR consists of five peaks, labeled waves I-V. The first peak, or wave I, originates from the action potential in the auditory nerve, wave II from the ipsilateral cochlear nucleus, wave III from the contralateral superior olivary complex, wave IV bilaterally from the lateral lemniscus, and wave V from the lateral-most portion of the contralateral IC (Melcher & Kiang, 1996). The mouse ABR is primarily generated by waves I-III and the largest amplitude waves are waves I and II (Miko et al., 2008). Previous studies of ABRs in mice have focused on measuring the amplitudes and latencies of waves I-III due to their robust response.

In humans, the neurodiagnostic ABR consists of five peaks, waves I-V, which occur within the 6 millisecond period following a high-intensity, transient stimulus. There are various clinical applications for the neurodiagnostic ABR. It can be used to diagnose eighth nerve or auditory brainstem dysfunction, as well as to monitor the status of the auditory system during surgery through intraoperative monitoring. The neurodiagnostic ABR is influenced by conductive and sensory hearing loss; therefore, it can be used as a way to test for type of hearing loss. Additionally, the ABR is a tool to

estimate auditory sensitivity in populations unable to provide a behavioral response (Hall, 2007).

In mice, wave I is the largest wave; in contrast, wave V is the largest wave in a normal human ABR. Wave V is much more difficult to determine in mice due to its tendency to blend in to the noise floor. Wave V in humans is generated by fibers of the ascending portion of the lateral lemniscus terminating in the IC and has contralateral contributions. Because wave V is receiving contributions from multiple pathways traveling up the brainstem, there is a natural amplification in the auditory system when the pathways reconnect. Wave I is generated by the distal portion of the auditory nerve and is typically a smaller response, as its generator is located only on the side ipsilateral to stimulation. In ABR recordings, peak amplitude may be 1  $\mu$ V, but it is considerably smaller in most cases (Burkard, Don, & Eggermont, 2007).

High intensity stimuli at any frequency will activate more basal portions of the cochlea due to the asymmetric shape of the traveling wave. The latency is shorter when the base of the cochlea is activated since the traveling wave does not have far to travel before activation. Additionally, there is less of a synaptic delay when using high intensity stimuli regardless of frequency. Studies have shown that when click level is decreased, the ABR peak latencies will increase and the amplitudes will decrease (Burkard, Don, & Eggermont, 2007). Changes in latency are about equal for all peaks, and wave I will first be affected, causing a subsequent shifting of peaks (Ackley, Decker, & Limb, 2007). As the stimulus intensity continues to decrease closer to threshold, the waveform peaks will gradually disappear.

The interwave intervals (IWI) are a measure of the transmission time through the brainstem. This would mean that “the I-III IWI reflects the time taken to traverse the caudal brainstem, while the III-V IWI reflects the time taken to traverse the rostral

brainstem” (Burkard et al., 2007, p. 233). In subjects with no pathological processes present, the IWLs are expected to stay relatively stable even as intensity is changed, since the latencies of all waves should be shifting together. This important measure can help identify lesions in the auditory nerve or brainstem.

Click evoked ABRs are not capable of providing frequency specific threshold estimations. In theory, a click stimulates all frequencies equally; however the transducer shapes the response and may emphasize a particular frequency region more than others. For more frequency specific information, tone pip ABRs have been used clinically to provide pediatric auditory assessment. Tone burst stimulation at low- to moderate-intensity levels can result in ABR responses for threshold assessment that are typically within 20 dB of audiometric threshold.

The ABR has been used to assess the hearing function of many strains of mutant mice; however, little research has been done on mice with mutations of the Eph family. The most prominent study on this topic found that mice having mutations in EphA4 or ephrin-B2 have significantly altered levels and patterns of activation in the auditory brainstem following pure tone stimulation. The EphA4 mice had wave I amplitudes that were 54% smaller and wave II amplitudes that were 56% smaller than controls. Latencies of wave I and wave II were not significantly different in EphA4; however the latency of wave III was significantly longer. ABR thresholds were found to be 75% higher than controls. Based on these findings the authors concluded that, “EphA4 protein has pronounced effect on the magnitude of evoked activation in the auditory brainstem” (Miko et al., 2008, p. 43). The ephrin-B2 mice had wave I amplitudes that were 38% smaller than controls which suggests an auditory nerve and/or cochlear nucleus deficit. Wave II amplitudes were not significantly different. The latency of wave I was unaffected; however, wave II had a significantly shorter latency, whereas wave III had a

significantly longer latency. Thresholds in ephrin-B2 mice were found to be 20% higher than controls. Based on these findings, they concluded that the Eph proteins may be expressed in cochlear neural and non-neural regions and have involvement in maintenance of ion concentrations in the cochlea as well as structural development. The ABR changes also suggested “a poorly developed auditory nerve-cochlear nucleus junction...abnormality in the superior olivary complex...[and] slower neural conduction time through the auditory pathway may be an indication of the integrity of myelin” (Miko et al., 2008).

Topographic maps are evident in the auditory system as well as other sensory systems and rely on accurate transmission from the periphery to the central nervous systems. EphA4 and ephrin-B2<sup>lacZ</sup> mice have shown distinct afferent layers in the lateral superior olivary complex (LSO) before the onset of hearing. By postnatal day 8, these layers project to cover the range of frequencies in the ipsilateral and contralateral central nucleus of the IC (CNIC) and will sharpen and become more precise by the time hearing function emerges. Both EphA4 and ephrin-B2 have a strong presence in the LSO and dorsal nucleus of the lateral lemniscus as well as other locations along the brainstem. They are also expressed in discrete patches before auditory experience within the external cortex of the inferior colliculus (ECIC). Tonotopicity that begins in the mouse cochlea is continued to the LSO and CNIC. Differences between the two strains were seen in the way the expression gradients declined over time (postnatal day 0 to day 12), but at birth they both displayed an apparent gradient which became “more flattened or homogenous” with increasing auditory experience (Gabriel et al., 2011, p. 191). They concluded that the Eph-ephrins are capable of positional cues to allow development and function of the auditory system, which occurs similarly to other sensory systems with topographic mapping.



There are a considerable number of Eph genes, and damage to a single gene may or may not show significant effects. A study by Miko et al. (2008) found that EphA4 and ephrin-B2 changed the response of ABR measures to pure tones that resulted in increased ABR thresholds and changes in latency and amplitude measures, suggesting significant roles of this single gene in normal hearing. It is useful to study mice that have the ephrins or Eph receptors knocked out because they “have been shown to display phenotypic responses that correlate with anatomical defects” (Howard et al., 2003).

One study by Miko et al. (2007) of mice lacking Eph proteins looked at *c-fos* activation levels, an early gene that is correlated to activation of neurons. Pure tones were presented to the mice and within 15 minutes the mice were anesthetized for removal of the brain. Sections of the brainstem were prepared for quantitative and topographic analysis of the cells in the dorsal cochlear nucleus (DCN) and the medial nucleus of the trapezoid body (MNTB) under a microscope to determine the activation levels of *c-fos* as well as the position and spread of the activated frequency band. The DCN and MNTB are representative of two separate auditory pathways. Mice with mutations of EphA4 and ephrin-B2 were utilized in this experiment. They found that mice lacking EphA4 had changes in magnitude of activation levels in the DCN and MNTB, and mice lacking ephrin-B2 only had changes in magnitude of activation in the MNTB, but not the DCN. The researchers found that “ephrin-B2 protein is an important local cue for correct targeting in the developing central nervous system” (Miko et al., 2007). The differences in effects on activation levels between mice lacking the ligand and mice lacking the receptor indicate that effects on topography occur even with partial loss of the ligand. Based on gradients of these proteins being observed in the IC, the authors propose the continued importance of these proteins further up the brainstem.

Mice lacking function of the EphB2 receptors had severe malformation of the semicircular canals and reduced amount of endolymph, which resulted in chronic circling behaviors as evidence of vestibular system dysfunction. Mice with an absence of EphB2 and EphB3 receptors showed delays of the fibers crossing the midline, although they eventually projected to their targeted structure. The cell bodies and fibers in the efferent pathways were traced with DiI fluorescent, a lipophilic diffusible dye. The ipsilateral and contralateral pathways and cell bodies were found to have mutant pathways to the semicircular canals that were disturbed and often had atypical caudal extensions (Cowan, Yokoyama, Bianchi, Henkemeyer, & Fritzsche, 2000).

Studies have shown that ephrin-B2 and ephrin-B3 are expressed in the adult mouse cochlea, more specifically in the modiolus. Ephrin-B2 is also expressed in the neurons of the spiral ganglion, organ of Corti cells, the stria vascularis, and in a layered pattern in the cochlear duct (Cowan et al., 2000; Pickles et al., 2002). Mutations of EphB1 and EphB3 resulted in alterations of distortion product otoacoustic emissions amplitudes, suggesting their importance in normal cochlear function. Mice deficient in these proteins showed significantly decreased measures of amplitude at two months of age as compared to wild-type littermates. This compromised peripheral auditory function was not apparent in mice deficient in EphB2 and ephrin-B3 which indicates that although they are involved in development of the auditory system, they are not necessary for outer hair cell function (Howard et al., 2003).

Based on the literature review, it is expected that there will be changes in ABR latency, amplitude, and threshold measures in the mutant mice as compared to the wild-type mice. The absolute latencies and IWLs will be analyzed to determine effects on peripheral and central transduction time. Amplitudes of wave I will provide a measure of the peripheral function. Finally, the primary focus of this study will be on auditory

thresholds to provide an estimate of actual hearing sensitivity. Therefore, the role of the current study is to determine the functional consequences that occur with altered connectivity in Eph-ephrin mutant mice when tested with the ABR.

## Methodology

The mice used in this study were born and bred in an animal housing facility in the Health and Human Services building at James Madison University. The subjects in this study consisted of 27 mice from several different genetic groups. Mice were genotyped by PCR from tail samples. The control group consisted of 10 wild type mice from the C57BL/6J strain (Jackson Laboratories, Bar Harbor, ME) (5 females, 5 males) that were tested at approximately 75 days old. Three strains of mutant mice (provided by Dr. Mark Henkemeyer) were utilized in this study: ephrin-B3<sup>lacZ</sup> (EB3<sup>lacZ</sup>), ephrin-B3<sup>null</sup> (EB3<sup>null</sup>), and EphA4<sup>lacZ</sup>. In control of wild-type animals, ephrin-B3 is expressed in the cochlear nucleus, superior olivary complex, and lateral lemniscus, with no expression in the central nucleus of the IC. The EB3<sup>null</sup> <sup>-/-</sup> (n=3) are true knockouts, as they completely lack the ephrin-B3 protein. In normals, EphA4 is heavily expressed in the central nucleus of the IC. In the case of the lacZ mutants, the gene is not entirely knocked out, but part of it has been removed so that it is incapable of retrograde signaling. The kinase is deleted and a marker is inserted which encodes the enzyme  $\beta$ -galactosidase so that protein expression activity can be tracked (Gabriele, M., personal communication, 2010 & Gabriele et al., 2011). The mice were housed in a BioZone MiniSmart Rack System with ad libitum food and water and HEPA-filtered air into each cage. All experimental procedures were performed in compliance with the National Institutes of Health "Guide for the Care and Use of Laboratory Animals" (NIH publications No. 80-23, revised 1996) and received prior approval by the Institutional Animal Care and Use Committee at James Madison University (Gray IACUC Protocol #A04-09, "Hearing Tests in Mice")

Testing was performed in wild-type and mutant adult mice at 2-3 months of age (average=61.9 days), before any documented age-related hearing loss in the control C57BL/6J strain (Ehret, 1976). The mice were lightly and momentarily anesthetized with

5% isoflurane and then injected IP with 150 mg/kg ketamine and 30 mg/kg xylazine. Each mouse received additional 1/3 doses as needed. The mice were placed in a sound-proof booth in a restraint on an Animal Blanket Control Unit to maintain body temperature during testing.

ABR measures were obtained by using three small subdermal needles: the noninverting electrode on the vertex, the inverting electrode on the mastoid of the test ear, and the ground electrode at the base of the neck. Electrodes were adjusted until the impedance values were 1k $\Omega$  or less. Braided electrode leads were then connected to a Tucker Davis RA4PA 4-channel Preamp.

A Y-shaped closed-tube sound delivery system measured the output of a TDT EC1 high-frequency electrostatic speaker with an Etymotic research ER-7C probe microphone (see Figure 1 for setup). Proper placement of the system was verified using the Dynamic Signal Analyzer and recording values of peak frequency, amplitude, and bandwidth of the stimulus during the highest-intensity neurodiagnostic ABR for each different stimulus for each mouse. Visual monitoring of the activity of the mouse throughout testing was achieved with an infrared camera. If high activity was occurring, additional 1/3 dose injections of ketamine/xylazine were administered. Following the conclusion of testing, the mice were allowed to fully recover from anesthesia before being returned back to their cages.

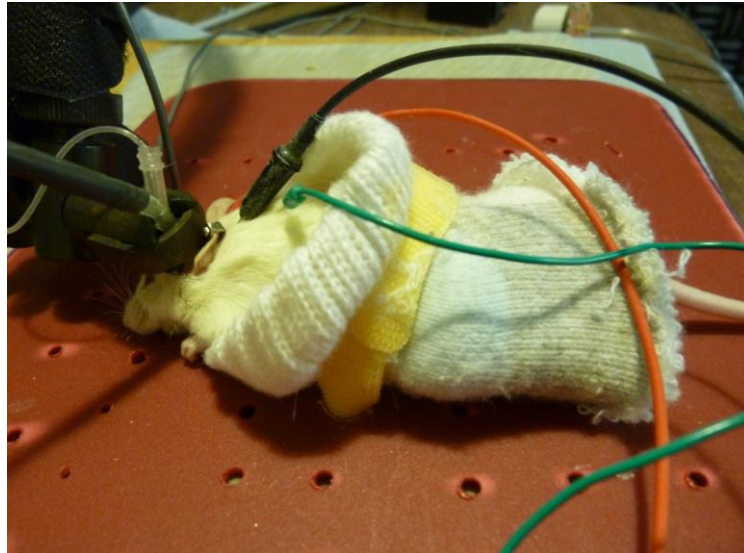


Figure 1. Setup for ABR measures with electrodes and transducer in place.

### **Calibration**

The loudest stimuli presented (nominally 90 dB on the BioSig system) were presented using the TDT SigGen software. The clicks were calibrated by finding their peak-equivalent sound pressure level through the process described below. Stimuli for the closed-tube delivery system were presented by a Tucker Davis Technologies TDT EC1 with the end of the closed tube sound delivery system placed into the end of a specially built artificial ear. The artificial ear was a plastic cavity with the expected volume found in the literature of an adult murine external auditory meatus.

The Agilent 35670A spectrum analyzer was first calibrated with a ¼ inch B&K 4939 microphone to a 1/4 inch coupler into a Bruel & Kjaer Model 4230 sound level calibrator to read 94 dB. The ¼ inch microphone was then connected to the artificial ear. No attenuator was attached to the microphone pre-amplifier. The amplitude switches on the Listen Inc amplifier were set to A1=20, A2=20, A3=0, and the polarization voltage was 200. The output of the TDT System3 - from the digital-to-analog converter, RP3, through a programmable attenuator, PA5, was connected directly to the TDT EC1 driver.

To calibrate the clicks, the Y-tube sound delivery system was placed against the artificial ear, in a way that was as similar as possible to the way the system was placed against the mouse meatus. The analog output of the ¼ inch microphone attached to the artificial ear was then connected to an Agilent 5461D digital oscilloscope. The peak-to-peak voltage of the nominally 90 dB click was recorded to be 116 mV. To convert this peak-to-peak voltage ( $V_{p-p}$ ) to dB ppeSPL, the dB SPL of a long 1 kHz pure tone (generated by the B&K 4230 calibrator and a long 1 kHz pure tone from SigGen) was noted on the spectrum analyzer at the same time the digital oscilloscope calculated the  $V_{p-p}$ . This was done for several intensity levels. A regression line of the stimulus intensity as a function of  $V_{p-p}$ , with both expressed in dB, revealed a linear function with slope of 1 and intercept of 14. Thus, 14 dB were added to the BioSig intensity levels for correction to dB ppeSPL for the clicks.

Calibrations were also performed for 8 kHz and 12 kHz pure tones of 1 s-duration each presented at a nominal (uncalibrated) level in the BioSig software of 90 dB. The 8 kHz tone output was 106 dB SPL and the 12 kHz tone output was 108 dB SPL. Thus, 16 and 18 dB was added for correction to dB SPL for the 8 and 12 kHz tone pips, respectively. Using the probe-tube system attached to the artificial ear, the same calibrations were run as done at the beginning of each recording from the mice. That is, the ER7C probe was connected to the Spectrum Analyzer and results from a peak-hold average during presentation of the loudest stimulus was recorded. Into the artificial ear, these measurements revealed peaks of 49, 68, and 79 dB for the clicks, 8 and 12 kHz, respectively. The peak energy for the click occurred at about 3.1 kHz and had a band level of 59 dB (200-20000 Hz). The peak of the tone pips occurred at the expected frequency.

In summary, clicks were calibrated with the peak-to-peak equivalent sound pressure level (dB ppeSPL). Pure tones were calibrated with the simple sound pressure level (dB SPL). Throughout the following results, 'dB' will mean dB ppeSPL when used as the intensity of clicks, and 'dB' will mean dB SPL when used as the intensity of tone pips.

### **Protocol**

Tucker Davis Technology (Achula FL) BioSig software was used to obtain ABR measures. The sample rate was 24498/s. The gain of the TDT RA4 Medusa Base Station neurophysiological amplifier was 20. The recorded response was digitally filtered from 300 to 3000 Hz with an artifact rejection of 20. A 10 ms time window produced 244 samples per sweep. The ABR protocol was arranged to begin at an uncalibrated level of 90 dB and decrease in intensity to 20 dB. The nominal intensity levels presented were 90, 70, 60, 55, 50, 45, 40, 35, 30, 25, and 20 dB. A total of four waveforms with 100 sweeps each were obtained at each intensity level (condensation, rarefaction, condensation, rarefaction). Condensation and rarefaction waveforms were summed to eliminate the cochlear microphonic and ensure reproducibility. At the end of the runs, another set of waveforms at 90 dB was obtained to guarantee integrity of the signal at the end of testing.

Three stimuli were presented to each mouse; broadband clicks, 12 kHz tone pips, and 8 kHz tone pips. Presentation of clicks and the 12 kHz tone pip were randomized, and if the mouse remained sedated, the 8 kHz tone pip was presented as well. The clicks were 0.1 ms in duration, while the tone pips were 5 ms in duration (with 0.5 ms Blackman windowed rise/decay times. Stimulation occurred at a rate of 39.1 cps.

Based on normative data on hearing sensitivity in mice, testing was performed at 2-3 months of age to reduce the effects of age-related hearing loss. At this age,



behavioral testing in C57BL/6J mice has shown thresholds at 8 kHz of 35 dB SPL (Miko et al., 2007). Tone bursts of 8 kHz and 12 kHz were chosen since they lie within the normal range of hearing in adult mice and will provide frequency specific information about hearing sensitivity in the optimal range of hearing (Ehret, 1976). The ABR thresholds for all four groups of mice were compared and analyzed for statistical significance.

Threshold was defined in dB as halfway between the last level a response was obtained and the first level that no response was present. Latencies and amplitudes of prominent peaks in the ABR were obtained using Matlab software written by the thesis advisor. Latency was calculated in milliseconds from the stimulus onset to the peak of the wave and corrections were made for tube length. The latencies of wave I to wave V were obtained at each intensity level until threshold was reached. Peak amplitude measures were obtained for wave I and wave II which have been shown to have the largest amplitude in mice. The amplitude was calculated from the peak of the wave to the following trough.

Latency-intensity functions (LIFs) were assembled for waves I and V and were compared for each group of mice. Studies have shown that wave I in an ABR is derived from the distal portion of the auditory nerve, and that the LIF will be similar for all ABR peaks. LIFs are “dependent on processes limited to the cochlear hair cell-auditory nerve fiber synapse and the auditory nerve up to the axon hillock, where the action potential is initiated,” and is considered to be a peripheral process (Burkard, Don, & Eggermont, 2007, p. 235). Research has suggested that when performing a click-evoked ABR at high intensities in humans, the cochlear base is stimulated, whereas clicks near threshold are generated by frequencies in the region of 1-2 kHz. This latency effect is supported by the fact that the traveling wave has a delay as it moves towards the apex. Due to this

phenomenon, there is an apical shift in the primary place of stimulation along the basilar membrane which roughly produces a 1 ms latency increase (Hall, 2007).

## Results

Statistical analyses utilized independent samples t tests, one-way ANOVAs with post Hoc tests, and analyses of covariance. P values less than .05 are considered significant. Table 1 shows the mean thresholds for the clicks, 8 kHz tone pip and 12 kHz tone pip for each mouse strain tested and Figure 2 is a box plot of thresholds by group.

Group	Stimulus	Mean Threshold (dB)	N	Std. Deviation (dB)	Effect Size (Cohen's d)
C57BL/6J	Click	57	10	8	
	8 kHz	58	10	7	
	12 kHz	51	10	8	
EphA4	Click	64	8	17	0.9
	8 kHz	60	7	22	0.3
	12 kHz	71	8	19	2.5
Ephrin-B3 <sup>Lz</sup>	Click	75	4	22	2.3
	8 kHz	68	3	25	1.4
	12 kHz	76	3	25	3.1
Ephrin-B3 <sup>null</sup>	Click	72	3	6	1.9
	8 kHz	75	2	9	2.4
	12 kHz	98	1		5.9

Table1: Average thresholds for the click, 8 kHz tone pip and 12 kHz tone pip. The dB for clicks is dB ppeSPL and the dB for tones is dB SPL

There were significant differences between groups for the click thresholds ( $F_{3,21}=4$ ,  $p=.026$ ). The C57BL/6J group and EphA4 group were the same. The C57BL/6J group was statistically different from the ephrin-B3<sup>Lz</sup> group and the ephrin-B3<sup>null</sup> group. The ephrin-B3<sup>Lz</sup> group and the ephrin-B3<sup>null</sup> group are essentially the same, and the ephrin-B3<sup>null</sup> and the EphA4 group are the same as well. The EphA4 group was statistically different from the ephrin-B3<sup>null</sup> group.

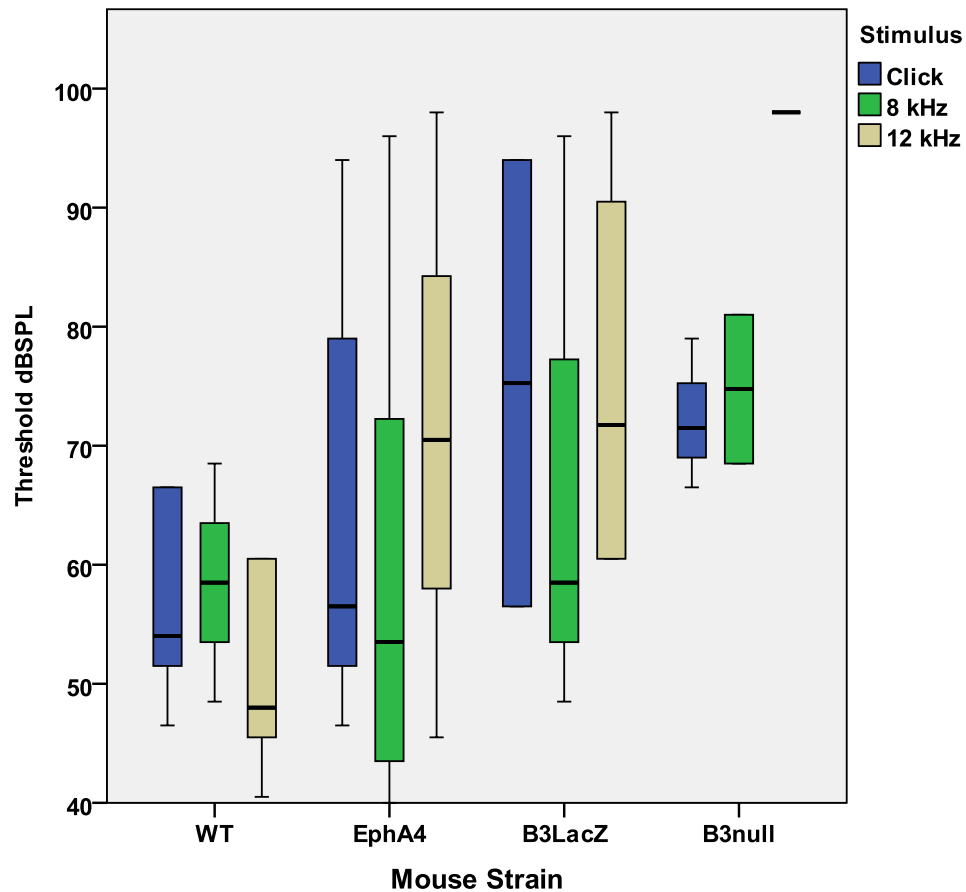


Figure 2. Box plot of thresholds by group.

There were no significant differences between groups for the 8 kHz stimulus ( $F_{3, 18}=1$ ,  $p=.520$ ). There was a significant difference between groups ( $F_{3, 19}=9$ ,  $p=.001$ ) in the 12 kHz thresholds. Post Hoc tests showed that the ephrin-B3<sup>Lz</sup> group is not different from the ephrin-B3<sup>null</sup> group or the EphA4 group. The C57BL/6J group showed significant differences from all other groups. The ephrin-B3<sup>Lz</sup> group and the EphA4 group were not statistically significant from each other. The ephrin-B3<sup>Lz</sup> is almost different from the ephrin-B3<sup>null</sup> group. The ephrin-B3<sup>null</sup> group is statistically different from the EphA4 group.

There were no significant differences for any group in the calibrated levels for all stimuli tested. However, the differences were most evident with the 12 kHz stimulus.

Click ( $F_{3,23}=1$ ,  $p=.386$ ), 8 kHz ( $F_{3,21}=1$ ,  $p=.580$ ), and 12 kHz ( $F_{3,23}=2$ ,  $p=.145$ );

thresholds for all groups had no significant effects from calibration levels.

The latency-intensity functions for wave I (see Figure 3) show clear trends with the wild-type group having the shortest latencies, the EphA4<sup>Lz</sup> having the next longest latencies, and some overlap between the ephrin-B3<sup>Lz</sup> and ephrin-B3<sup>null</sup> groups which had the longest overall latencies. The response is clearly under stimulus control as seen by the decreasing latencies as intensity of the clicks increases. Statistical evaluation with ANCOVA shows effects on Wave I latency (see Table 2 for latency measures) due to mouse strain ( $F_{3,111}=21$ ,  $p<.001$ ) and to stimulus intensity level ( $F_{1,111}=39$ ,  $p<.001$ ). Similar effects were seen for the 8-and-12 kHz tone pips (all  $p$  values less than .001 for effects of mouse strain and for stimulus intensity for both frequencies of tone pips).

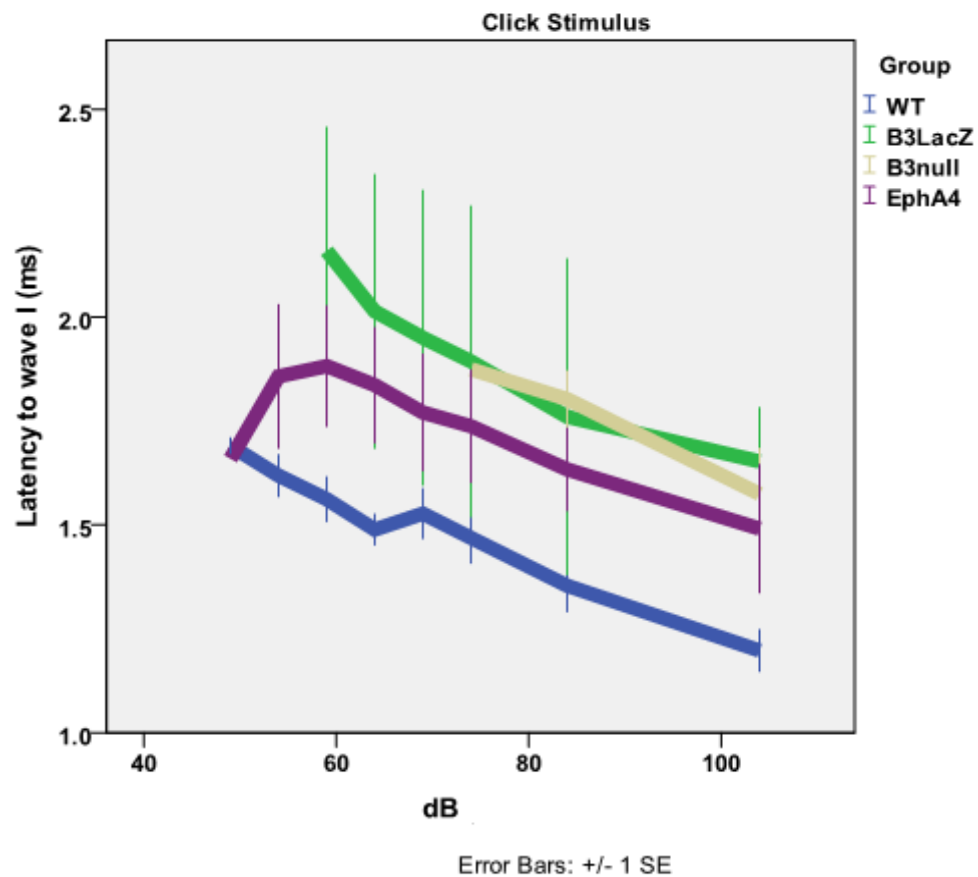


Figure 3. LIFs for wave I with a click stimulus. Intensity levels are measured in dB ppeSPL.

Group	Stimulus	Mean Wave I Latency (ms)	N	Std. Deviation (ms)	Effect Size (Cohen's d)
C57BL/6J	Click	1.20	10	.16	
	8 kHz	1.58	10	.25	
	12 kHz	1.62	10	.15	
Ephrin-B3 <sup>Lz</sup>	Click	1.65	4	.26	2.8
	8 kHz	2.24	3	.50	2.6
	12 kHz	2.15	4	.31	3.5
Ephrin-B3 <sup>null</sup>	Click	1.57	3	.19	2.3
	8 kHz	1.73	2	.24	0.6
	12 kHz	1.11	1	--	-3.4
EphA4	Click	1.49	8	.44	1.8
	8 kHz	1.79	7	.38	0.8
	12 kHz	1.66	8	.37	0.3

Table 2: Average wave I latencies for each mouse group at 104 dB ppeSPL for the clicks, 106 dB SPL for the 8 kHz tone pip, and 108 dB SPL for the 12 kHz tone pip.

The discussion will be about wave V because of its relevance to the IC. Wave V latency-intensity functions for the click (see Figure 4) showed no significant differences by mouse strain ( $F_{3,91}=2$ ,  $p=.129$ ) and a significant effect of stimulus intensity ( $F_{1,91}=4$ ,  $p=.059$ ). Wave V latency-intensity functions for the 8 kHz tone pip (see Figure 5) showed significant mouse strain differences ( $F_{3,81}=5$ ,  $p=.002$ ) and no significant stimulus intensity effects ( $F_{1,81}=2$ ,  $p=.148$ ). The 12 kHz tone pip had overlapping latency-intensity functions for the C57BL/6J and EphA4<sup>Lz</sup> groups with significantly longer latencies for the ephrin-B3<sup>Lz</sup> group (see Figure 6). There were significant effects by mouse strain ( $F_{2,98}=26$ ,  $p=.000$ ) and no effects by stimulus intensity ( $F_{1,98}=1$ ,  $p=.310$ ). There are a smaller number of mice in the ephrinB3<sup>null</sup> group due to the small number of mice that had measurable ABR waveforms. Average latencies for wave V can be seen in Table 3.

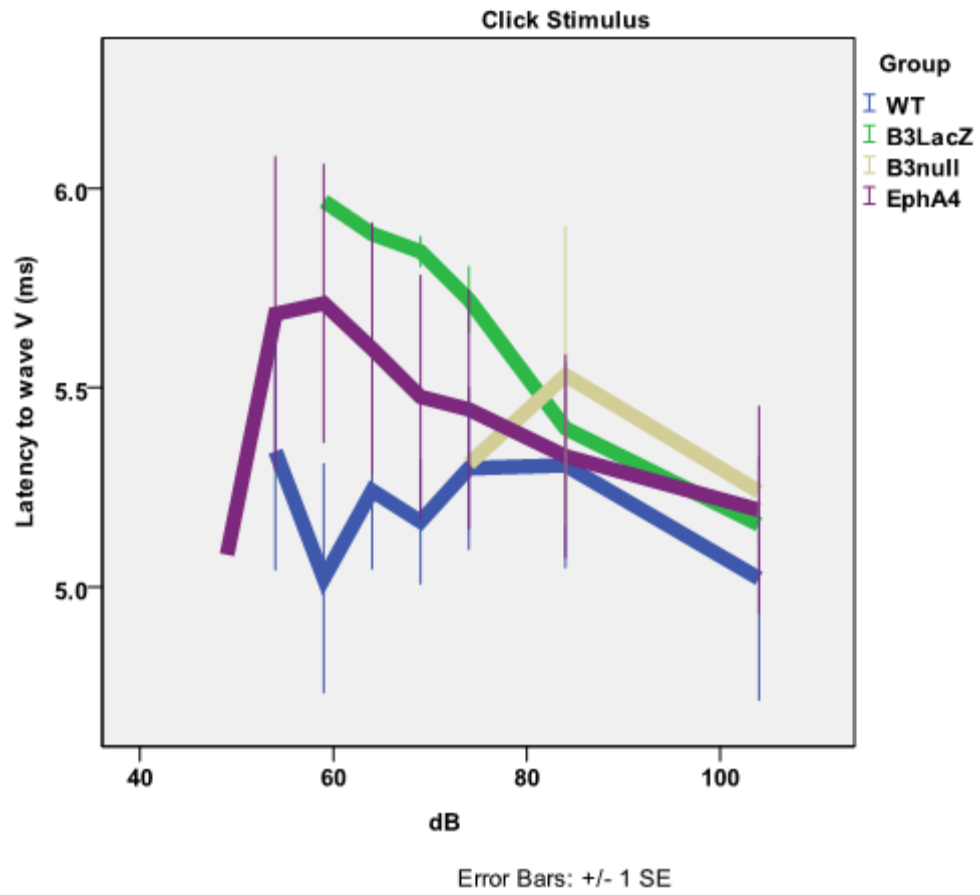


Figure 4. LIF for wave V with a click stimulus. Intensity levels are measured in dB ppeSPL.

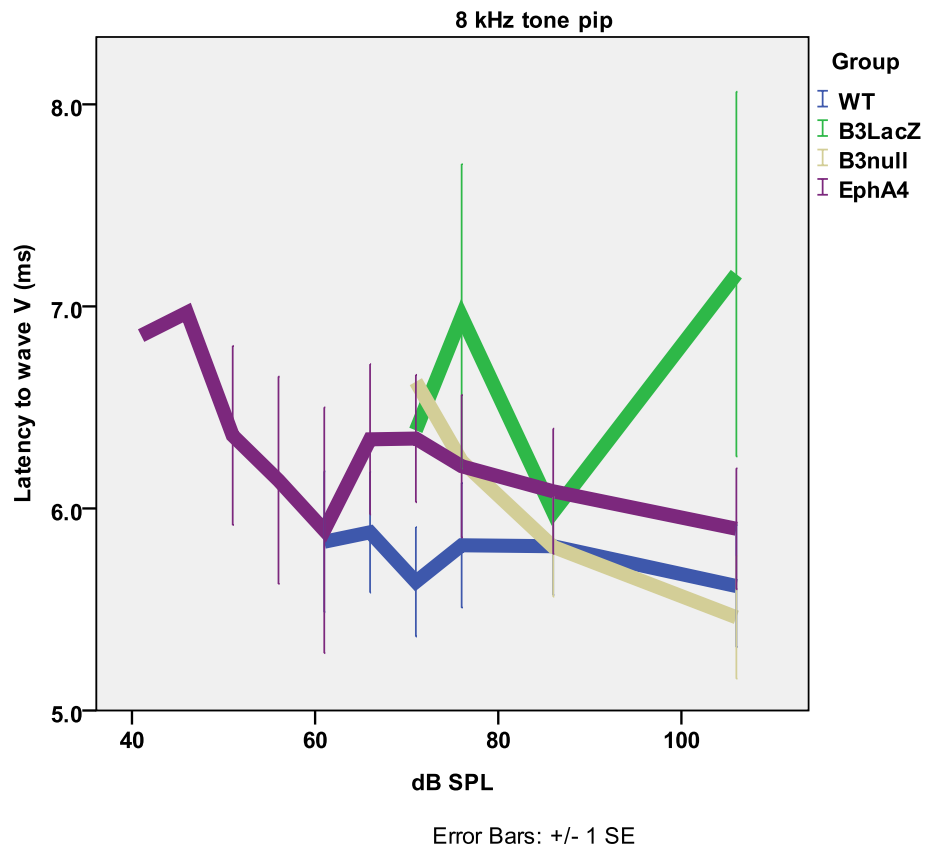


Figure 5. LIFs for wave V with an 8 kHz tone pip stimulus.



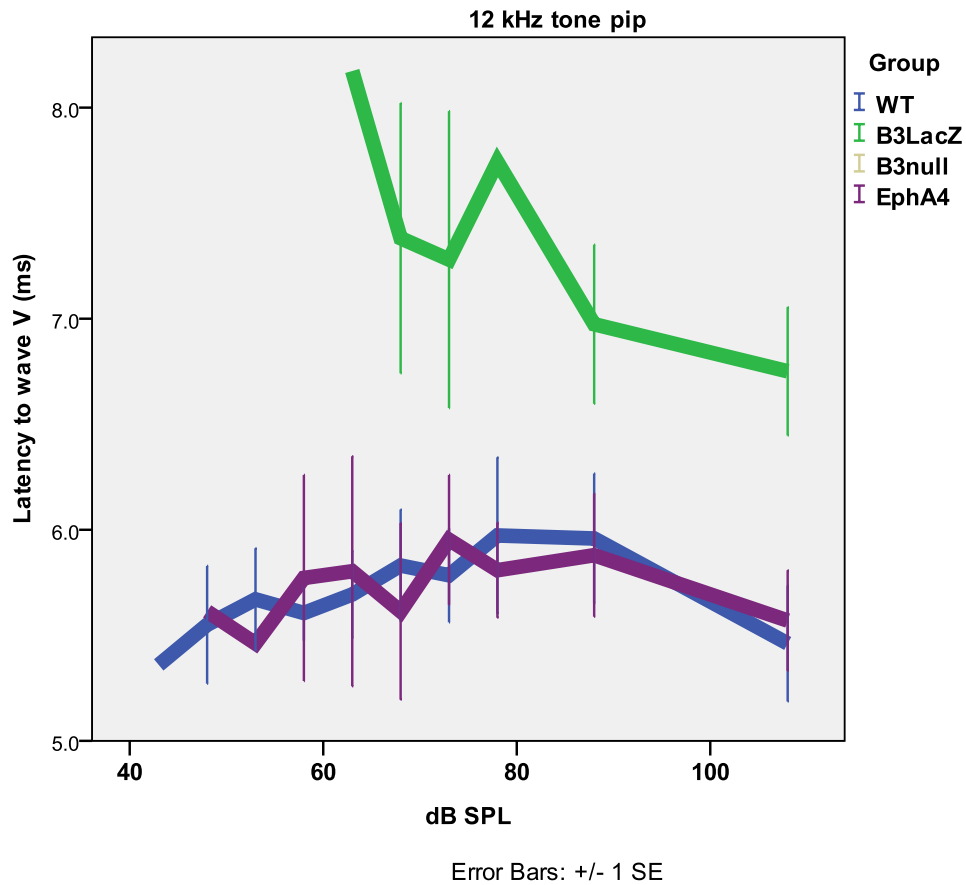


Figure 6. LIFs for wave V with a 12 kHz tone pip stimulus.

Group	Stimulus	Mean Wave V Latency (ms)	N	Std. Deviation (ms)	Effect Size (Cohen's d)
C57BL/6J	Click	5.02	10	1.0	
	8 kHz	5.62	10	1.0	
	12 kHz	5.46	10	.87	
Ephrin-B3 <sup>Lz</sup>	Click	5.16	2	.05	0.1
	8 kHz	7.16	3	1.6	1.5
	12 kHz	6.75	4	.61	1.5
Ephrin-B3 <sup>null</sup>	Click	5.24	2	.08	0.2
	8 kHz	5.46	2	.43	-0.2
	12 kHz	--	--	--	
EphA4	Click	5.19	7	.69	0.2
	8 kHz	5.90	7	.79	0.3
	12 kHz	5.57	7	.73	0.1

Table 3: Average wave V latencies for each mouse group at 104 dB ppeSPL for the clicks, 106 dB SPL for the 8 kHz tone pip, and 108 dB SPL for the 12 kHz tone pip.

The wave I-V IWIs for the C57BL/6J, ephrin-B3<sup>Lz</sup>, ephrin-B3<sup>null</sup>, and EphA4 groups were analyzed for significance. The wave I-V IWI for the clicks (see Figure 7) showed no significant differences by mouse strain ( $F_{3,91}=1$ ,  $p=.926$ ) or by intensity ( $F_{1,91}=1$ ,  $p=.682$ ). Wave I-V IWI for the 8 kHz tone pip (see Figure 8) showed significant mouse strain differences ( $F_{3,77}=4$ ,  $p=.019$ ) and no significant stimulus intensity effects ( $F_{1,77}=1$ ,  $p=.658$ ). The 12 kHz tone pip wave I-V IWI (see Figure 9) showed significant effects by mouse strain ( $F_{2,87}=18$ ,  $p=.000$ ) and no effects by stimulus intensity ( $F_{1,87}=3$ ,  $p=.105$ ).

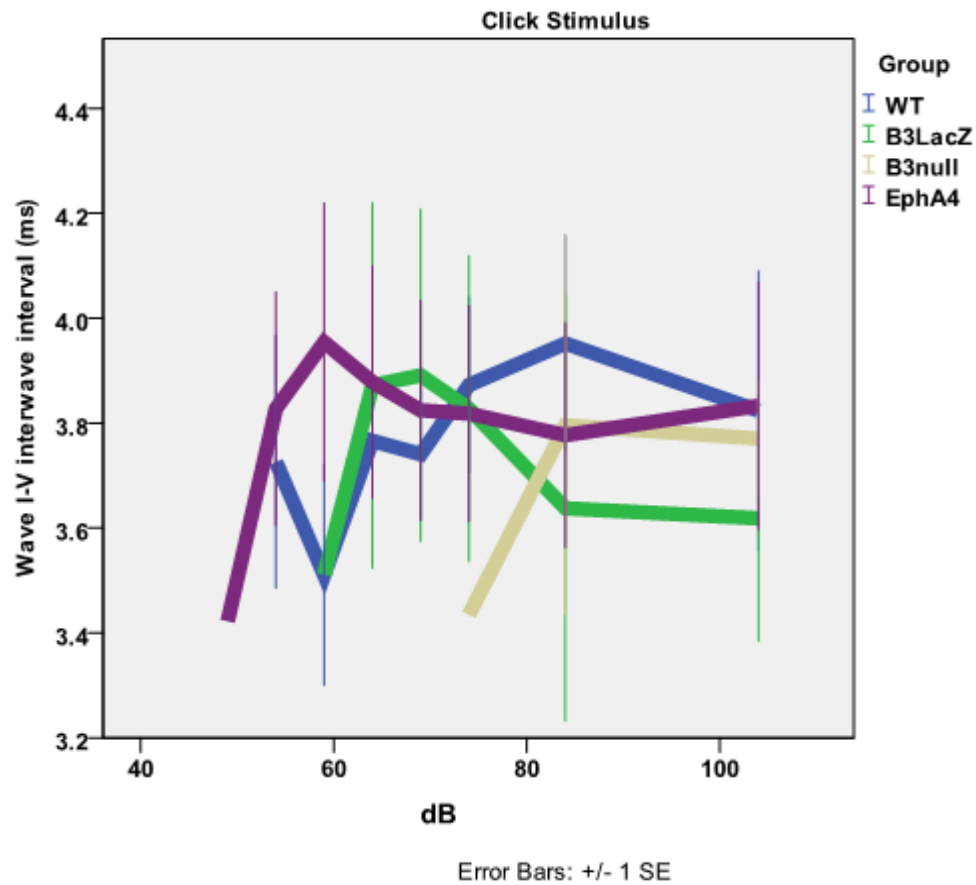


Figure 7. Wave I-V IWIs for the click. Intensity levels are measured in dB ppeSPL

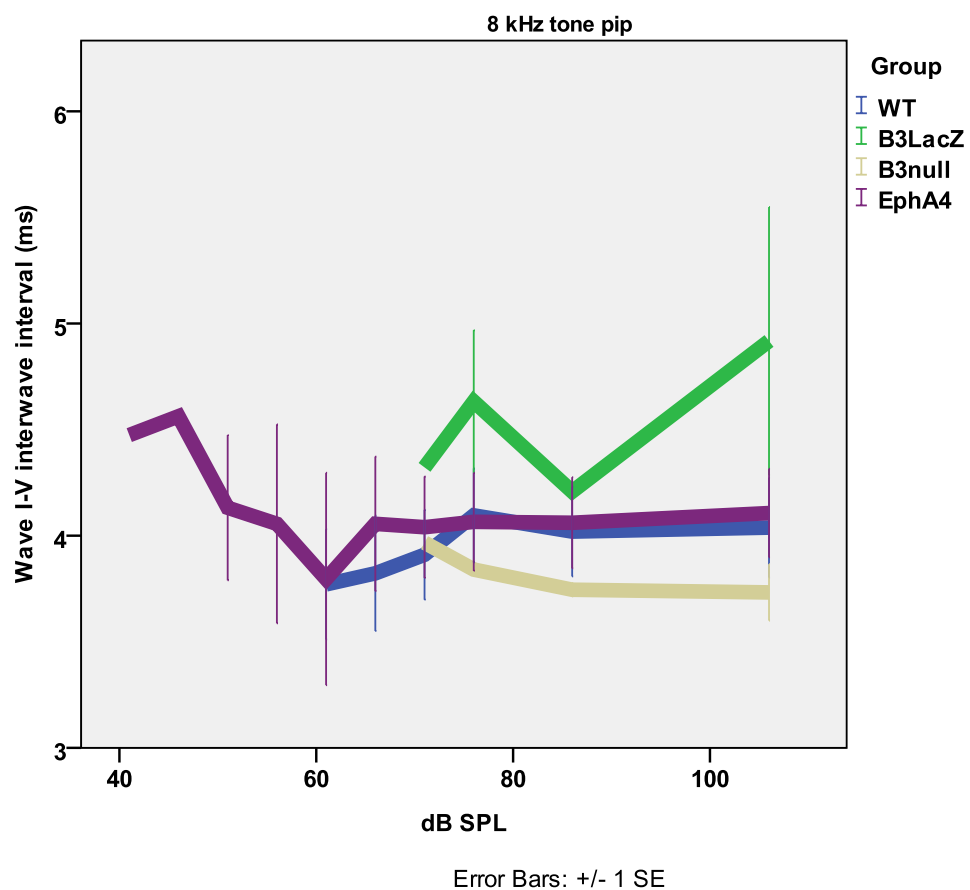


Figure 8. Wave I-V IWIs for the 8 kHz tone pip.

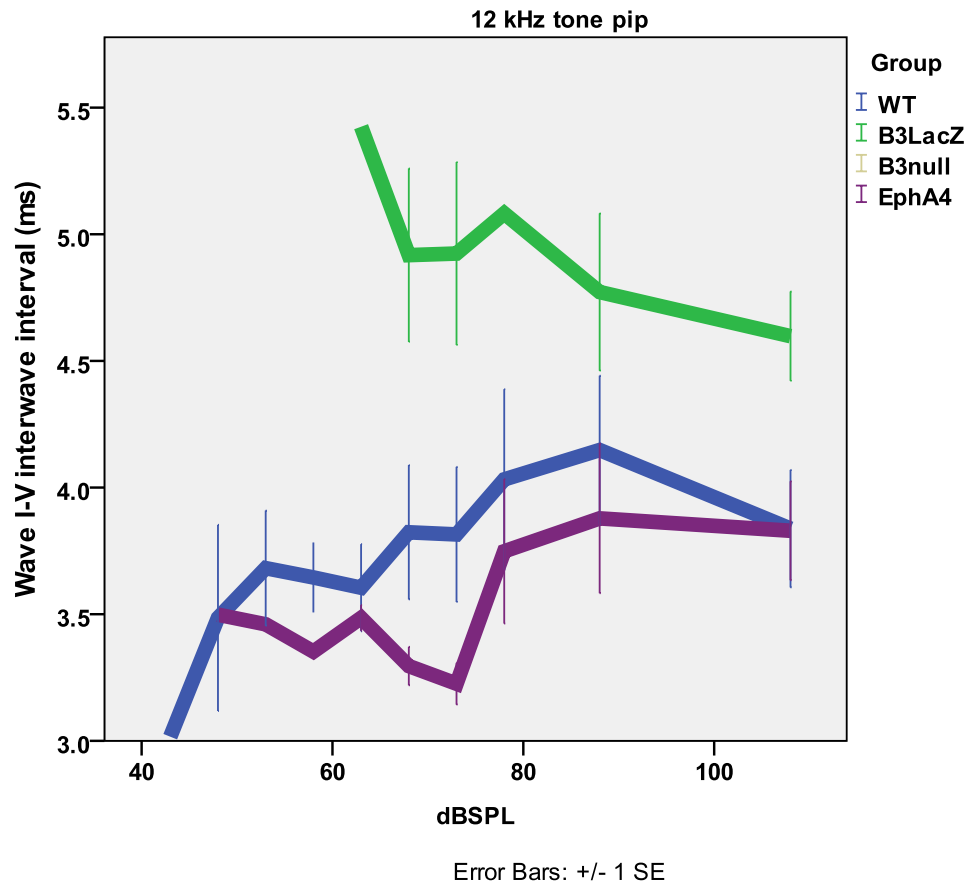


Figure 9. Wave I-V IWIs for the 12 kHz tone pip.

Amplitude of wave I was significantly different between groups for the clicks ( $F_{3, 115}=6$ ,  $p=.001$ ), 8 kHz tone pips ( $F_{3, 108}=3$ ,  $p=.023$ ), and the 12 kHz tone pips ( $F_{3, 111}=8$ ,  $p=.000$ ). See Figures 10, 11, and 12 for the amplitude/intensity functions for the click, 8 kHz, and 12 kHz tone pip, respectively. The wave I amplitude was significantly affected by the stimulus intensity across all test conditions ( $p<.000$ ). Refer to Table 4 for average wave I amplitudes for each group and stimulus.

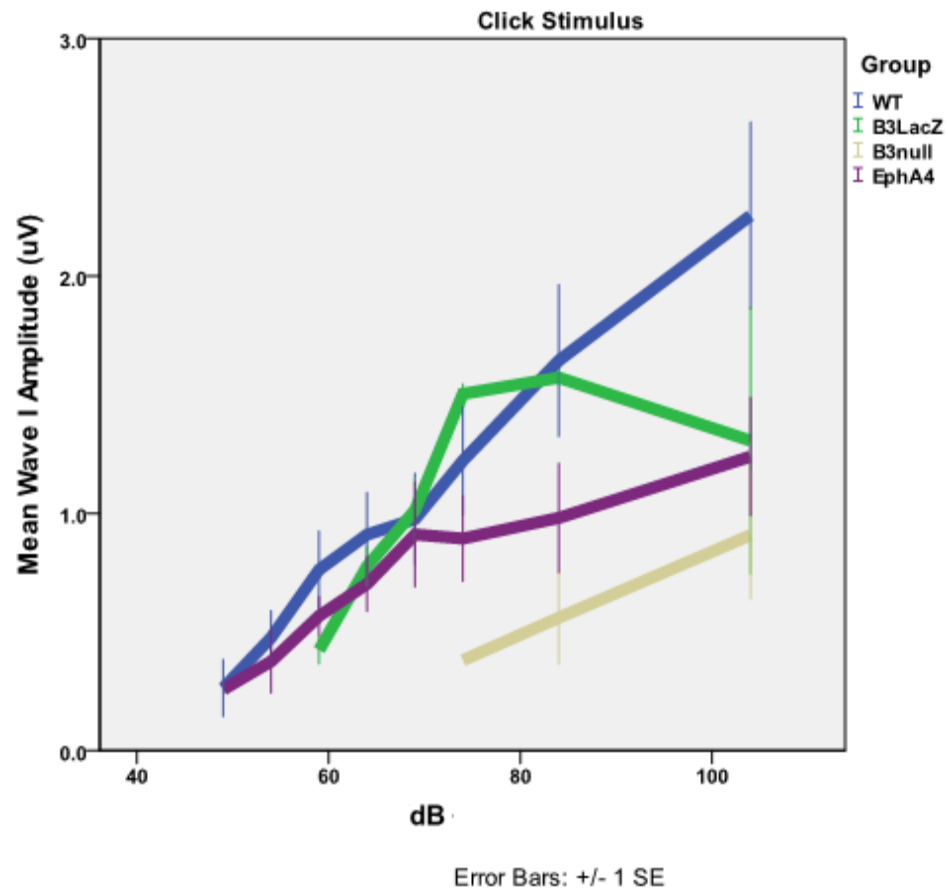


Figure 10. Wave I amplitude for the clicks. Intensity levels are measured in dB ppeSPL.

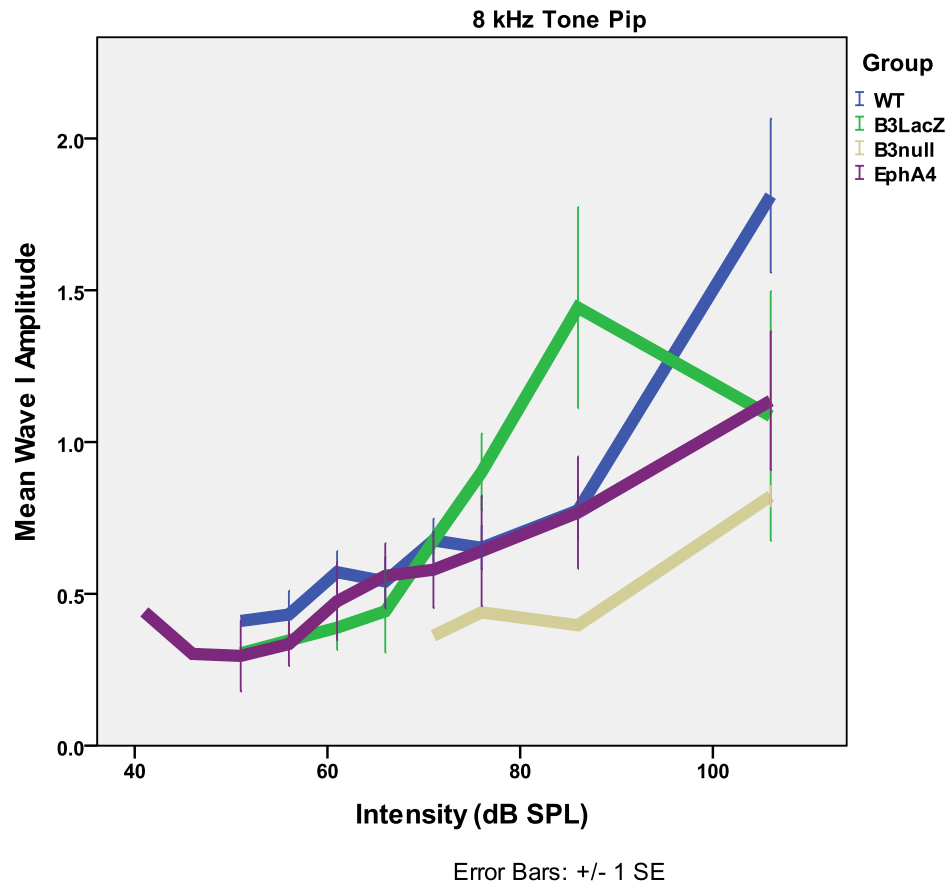


Figure 11. Wave I amplitude for the 8 kHz tone pip.

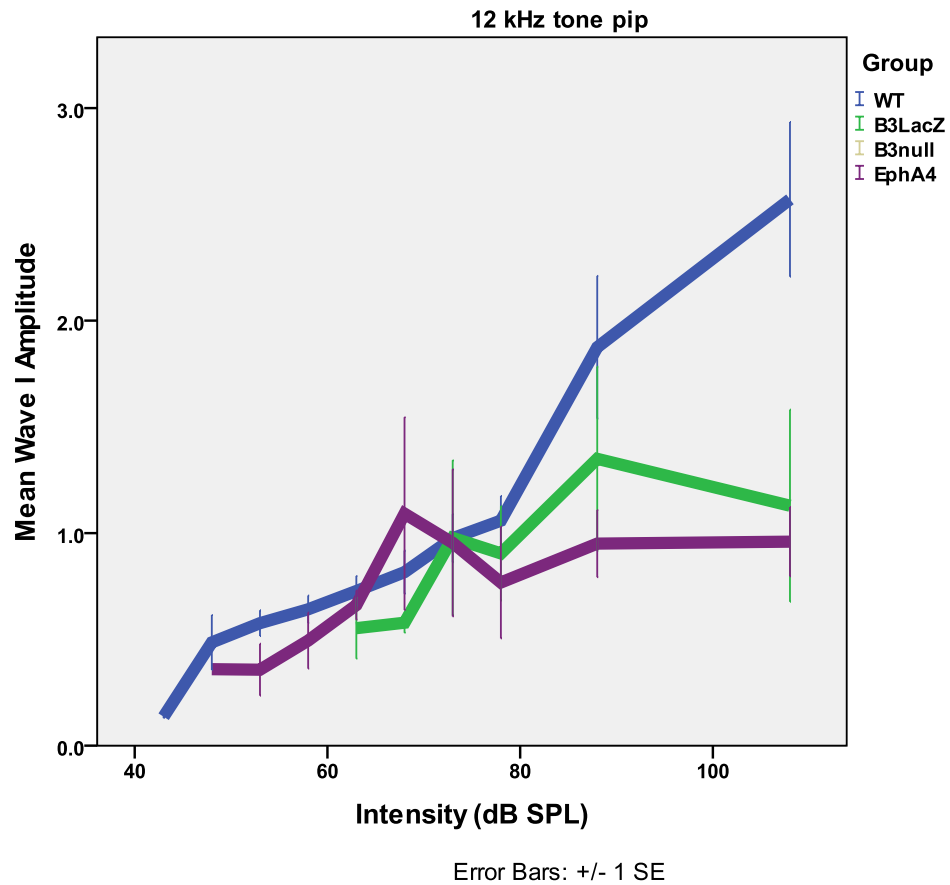


Figure 12. Wave I amplitude for the 12 kHz tone pip.

Group	Stimulus	Mean Amp I (μV)	N	Std. Deviation (μV)	Effect Size (Cohen's d)
C57BL/6J	Click	2.25	10	1.25	
	8 kHz	1.81	10	.80	
	12 kHz	2.57	10	1.15	
Ephrin-B3 <sup>Lz</sup>	Click	1.31	4	1.13	-0.8
	8 kHz	1.10	3	.71	-0.9
	12 kHz	1.13	4	.90	-1.3
Ephrin-B3 <sup>null</sup>	Click	.91	3	.47	-1.1
	8 kHz	.82	2	.05	-1.2
	12 kHz	.48	1	--	
EphA4	Click	1.24	8	.71	-0.8
	8 kHz	1.14	7	.61	-0.8
	12 kHz	.96	8	.46	-1.4

Table 4: Average wave I amplitudes for each mouse group at 104 dB ppeSPL for the clicks, 106 dB SPL for the 8 kHz tone pip, and 108 dB SPL for the 12 kHz tone pip.

The amplitude of wave II was significantly different between groups for the clicks ( $F_{2, 110}=4$ ,  $p=.015$ ), 8 kHz tone pips ( $F_{2, 117}=3$ ,  $p=.000$ ), and the 12 kHz tone pips ( $F_{2, 121}=13$ ,  $p=.000$ ). The wave II amplitude was also significantly affected by the stimulus intensity across all test conditions ( $p<.000$ ). Refer to Table 5 for average wave II amplitudes for each group and stimulus.

Group	Stimulus	Mean Amp II ( $\mu$ V)	N	Std. Deviation ( $\mu$ V)
C57BL/6J	Click	1.80	10	1.15
	8 kHz	2.53	10	1.52
	12 kHz	2.38	10	1.13
Ephrin-B3 <sup>Lz</sup>	Click	1.02	4	.66
	8 kHz	.69	3	.23
	12 kHz	.83	4	.27
Ephrin-B3 <sup>null</sup>	Click	.62	3	.21
	8 kHz	.58	2	.39
	12 kHz	.45	1	--
EphA4	Click	1.70	8	.91
	8 kHz	1.60	7	.78
	12 kHz	1.24	8	.47

Table 5: Average wave II amplitudes for each mouse at 104 dB ppeSPL for the clicks, 106 dB SPL for the 8 kHz tone pip, and 108 dB SPL for the 12 kHz tone pip.

Neurodiagnostic ABRs show graded effects of Eph-ephrin mutations. Figure 13 shows the grand average of all the recordings from all mice in each of 4 groups to the loudest (90 dB) clicks. The vertical scale is the same for all groups, although the different waves have been shifted vertically to avoid overlap. The amplitude of the ABR is decreased in the mutant groups and the prominent peaks are delayed relative to wild-type. The effects appear to be more significant at higher CNS levels, meaning that the later peaks are smaller and delayed. These data show that Eph-ephrin mutation play a significant role in the development of the afferent auditory system.



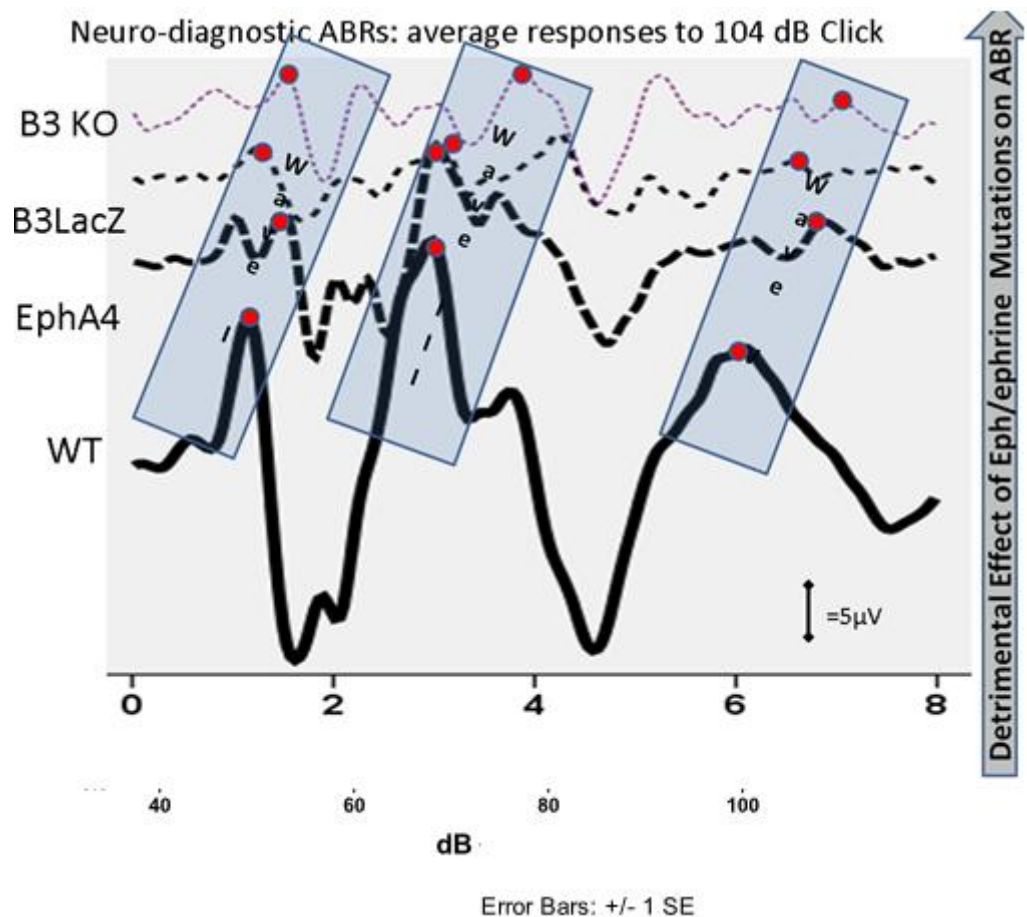


Figure 13. Neurodiagnostic ABRs in the 4 mouse strains tested to a 104 dB ppeSPL click.

### Some other analyses of group differences and calibrations

Significant differences in weight were seen, with the ephrin-B3<sup>Lz</sup>, ephrin-B3<sup>null</sup>, and EphA4 groups being statistically heavier than the C57BL/6J group ( $F_{3,23}=11$ ,  $p=.000$ ). The mutant groups did not show any significant differences in weight.

There were significant differences in age between all groups ( $F_{3,23}=5$ ,  $p=.010$ ). The EphA4 group had the oldest mice out of all that were tested. No group showed any effect of age on threshold.

Among the 3 mutant groups, there were no significant differences ( $F_{1,10}=2$ ,  $p=.235$ ) between homozygous-recessive (-/-) and heterozygous (-/+) genotypes, which is likely due to the small sample size.

Sex had no effects on thresholds, with  $p > .47$  over all groups and stimuli analyzed separately. More specifically, for clicks in the wild type group  $t_8 = .6$ ,  $p = .57$ ; for wild type 8 kHz tone pips  $t_8 = .63$ ,  $p = .55$ ; for wild type 12 kHz  $t_8 = .76$ ,  $p = .47$ ; 5 females had the same thresholds as 2 males in the EphA4 group with significance in all three stimuli above .76; among the other groups, only click thresholds in the ephrin-B3<sup>null</sup> group could be evaluated, and sex was not significant with  $p = .76$ .

Among the C57BL/6J group, there was no correlation between thresholds and stability of waveforms. In the mutant groups, the correlations were poor because of poor neurodiagnostic ABR waveforms at the highest intensity.

There were no effects on thresholds of the real-time calibrations from the probe tube during testing. The C57BL/6J group only correlations are all  $p > .05$ . Small sample sizes in the mutant groups makes this analysis too variable.

## Discussion

### Auditory Thresholds

There are clear differences in the thresholds, LIFs, amplitudes, and neurodiagnostic ABRs in Eph-ephrin deficient mice. This shows that the Eph-ephrin family of receptor/ligands plays an important role in the establishment of a fully functional auditory system. The true ephrin-B3<sup>null</sup> group seems to be most affected.

The clicks have most of their energy at an average of 3.2 kHz, according to the real-time calibrations performed during the testing of each mouse. Thus, the click thresholds are plotted at 3 kHz, and Figure 14 shows the physiological audiograms of the different groups from the ABR threshold data.

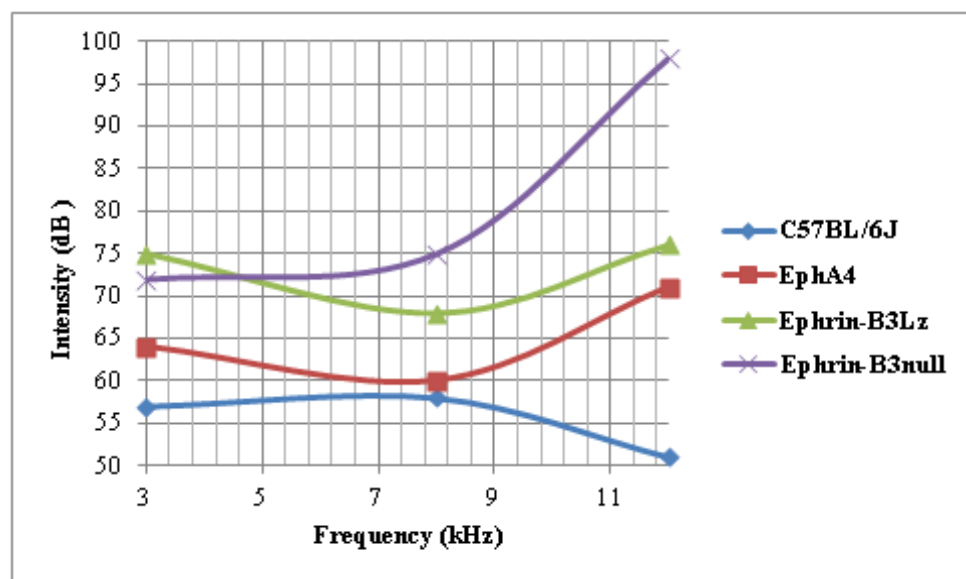


Figure 14. Physiological 'Audiograms' for all mouse strains tested (ABR thresholds versus spectral peak). Intensity levels are measured in dB SPL for tone pips.

Overall, thresholds were significantly different for test conditions with the click and 12 kHz tone pip between all four mouse groups tested suggesting an importance in the Eph receptors and ephrins in development of the auditory system. The 8 kHz tone pip showed no significant differences between groups, likely due only to variability. Figure 2

objectively shows similar trends in thresholds across groups at all frequencies. The 8 kHz stimuli were presented last, so individual differences in stability over time would have contributed to increased variability, and could likely have brought differences out of the range of significance at the .05 level. There were no differences across groups in our measure of preparation stability (correlation of loud clicks at the beginning and end of testing), but within-group variability in stability could still have caused the lack of significant difference at the 8 kHz. Calibrations performed during testing helped ensure that procedures remained consistent from mouse-to-mouse. Figure 15 shows a summary of Figure 2. Grand average thresholds for all stimuli are shown for each group. Thresholds deteriorate from the lowest thresholds seen in the C57BL/6J (or wild type) mice, to the EphA4 mice, Ephrin-B3<sup>Lz</sup> mice, and finally to the highest thresholds in the Ephrin-B3<sup>null</sup> mice.

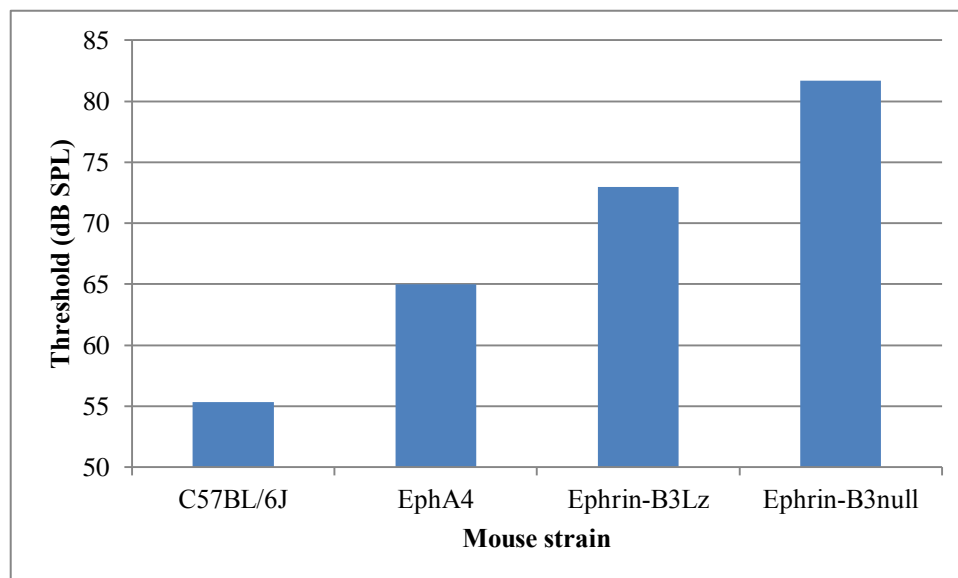


Figure 15. Average threshold for each mouse group across all stimuli tested.

Across all stimuli, the C57BL/6J mice had the lowest thresholds, as expected since they were assumed to have normal development of the auditory system. Overall the

average thresholds for the C57BL/6J were 55 dB SPL. The highest numbers of subjects were in this group equally distributed between males and females.

EphA4 receptors have expression in the central nucleus of the IC. This group of mice with these mutations had the largest number of subjects out of the collection of mutant strains tested. While one mouse died under anesthesia and was unable to be tested, all other subjects in this group had waveforms for each stimulus. Average thresholds across all stimuli were found to be 65 dB SPL.

The ephrin-B3<sup>Lz</sup> group is known to have some expression of ephrins within the cochlear nucleus, superior olivary complex, and lateral lemniscus, but no expression in the central nucleus of the IC. As such, they are not complete knockouts, but rather have part of the gene altered so it cannot signal. The waveforms for these mice had rather poor morphology; therefore, a mouse from the C57/6J group was run to ensure this finding was not due to an equipment malfunction. The average overall threshold was 73 dB SPL across all stimuli. One mouse was excluded from this group because it died under anesthesia during testing.

Ephrin-B3<sup>null</sup> mice had an overall average threshold of 82 dB SPL. This group was expected to have the poorest thresholds due to the extent of the auditory pathway mutations that occurred. It should be noted that the smallest number of subjects had recordable ABR waveforms in this group. Only three mice in this group had present waveforms for the click, two had 8 kHz waveforms, and one had a 12 kHz waveform. One mouse in this group was excluded because there were no measurable ABR waveforms for all stimuli even at the highest intensity level.

When looking at click thresholds across all groups, there was a 32% increase in threshold for the ephrin-B3<sup>Lz</sup> group, a 26% increase in threshold for the ephrin-B3<sup>null</sup> group, and a 12% increase in threshold for the EphA4 group. Analysis of the 8 kHz tone

pip showed a 17% increase in threshold for the ephrin-B3<sup>Lz</sup> group, a 29% increase for the ephrin-B3<sup>null</sup> group, and a 3% increase in the EphA4 group. Finally, the 12 kHz tone pip showed a 49% increase in threshold for the ephrin-B3<sup>Lz</sup> group, a 92% increase in the ephrin-B3<sup>null</sup> group, and a 39% increase in the EphA4 group. Therefore, the most significant effects on threshold occurred for the highest frequency presented, which was the 12 kHz tone pip. The 2008 Miko et al. study, found the EphA4 group to have 75% higher thresholds and the ephrin-B2 group to have 20% higher thresholds for clicks. While this study did not test any mouse from the ephrin-B2 strain, there were clear increases in threshold for the EphA4 group, just not as significant of an increase as found by Miko et al.

### **Neurodiagnostic ABRs**

The neurodiagnostic ABR data provides a good overall view of what is occurring along the brainstem across all mouse groups. Greater effects can be seen within the CNS which resulted in prolonged latencies, for example. The Eph-ephrins are therefore vital in developing a functional auditory system, and their roles extend well beyond the peripheral auditory system. This is a new area to consider in Eph-ephrin research and can provide information about how altered connectivity affects the ascending response. Previous studies did not investigate this far up the brainstem and only focused on peripheral consequences.

### **Latency Measures**

The latencies also showed effects across all stimuli and mouse strain, suggesting that Eph-ephrin mutations increased transduction times to wave I. Both ephrin-B3 groups (Lz and null) appeared to have some overlap in LIFs (see Figure 3), however the small number of ephrin-B3<sup>null</sup> mice with recordable waveforms make it difficult to compare to the larger group of ephrin-B3<sup>Lz</sup>. Of note, Figure 3 demonstrates LIFs that are essentially

parallel to one another, which can be indicative of a conductive hearing loss. However, this was the only instance where this parallel pattern occurred, and in the other test conditions the latency to wave I did not shift out uniformly. Additionally, this trend was not seen in the wave V LIF for any of the stimuli. If the hearing loss were truly conductive, this trend would be seen with both wave I and wave V.

When looking at latency to wave I for the clicks across all groups, there was a 38% increase in latency for the ephrin-B3<sup>Lz</sup> group, a 31% increase in latency for the ephrin-B3<sup>null</sup> group, and a 24% increase in latency for the EphA4 group. Analysis of the 8 kHz tone pip showed a 42% increase in wave I latency for the ephrin-B3<sup>Lz</sup> group, a 9% increase for the ephrin-B3<sup>null</sup> group, and a 13% increase in the EphA4 group. Finally, the 12 kHz tone pip showed a 33% increase in latency for the ephrin-B3<sup>Lz</sup> group, a 32% decrease in the ephrin-B3<sup>null</sup> group, and a 2% increase in the EphA4 group. In all instances, the wave I latency was found to increase, with the exception of the 12 kHz tone pip in the ephrin-B3<sup>null</sup> group where a shorter conduction time was noted; however, only one subject in this group had measurable waveforms. Once again this could likely be contributed to the poor morphology and high variability of the waveforms which made valid latency measures more difficult. Overall, the ephrin-B3<sup>Lz</sup> group had the greatest increases in wave I latency across all stimuli tested, suggesting deficits in the auditory nerve.

While this study set out to focus on wave V to determine effects on different amounts of expression in the IC, analysis revealed difficulty in finding a clear and consistent wave V in all mouse groups tested. Different electrode montages and additional channels were attempted to increase the amplitude of wave V, but analysis of these did not show any clear improvements in recordings. While there were no group differences in wave V LIF for clicks, there were differences between groups for the 8 kHz

tone pip, and longer latencies for the ephrin-B3<sup>Lz</sup>. Once again, the small number of mice in the ephrin-B3<sup>null</sup> group with measurable waveforms made it difficult to see clear trends in wave V LIFs. In fact, for the 12 kHz stimulus, there were no mice in that group that had a wave V measurable at decreasing intensity levels.

Wave V latency for the clicks was found to increase by 3% for the ephrin-B3<sup>Lz</sup> group, 4% for the ephrin-B3<sup>null</sup> group, and 3% for the EphA4 group. Analysis of the 8 kHz tone pip showed a 27% increase in wave V latency for the ephrin-B3<sup>Lz</sup> group, a 3% decrease for the ephrin-B3<sup>null</sup> group, and a 5% increase in the EphA4 group. Finally, the 12 kHz tone pip showed a 24% increase in latency for the ephrin-B3<sup>Lz</sup> group, and a 2% increase in the EphA4 group. There were no wave V peaks measurable in the ephrin-B3<sup>null</sup> group for the 12 kHz tone pip. Trends for wave V latency were less clear than for wave I latency; however, the ephrin-B3<sup>Lz</sup> group had the largest percent changes out of the 3 mutant groups. This suggests more significant effects of this strain on the IC. The same finding was true for the wave I latency, where the ephrin-B3<sup>Lz</sup> group had the largest increases in latency.

### **Interwave Intervals**

Analysis of wave I-V IWI provided a means of comparing conduction time from the brainstem up to the midbrain. Significant effects of groups were seen in conduction times for the 8-and-12 kHz tone pips, but not the click. There should not be an effect of intensity on this measure, because the IWI should take similar amounts of time regardless of the stimulus intensity, as expected. This study did not find any significant effects on the wave I-V IWI for intensity. When looking at the figures for the various stimuli tested, the click stimulus IWI showed a fair amount of overlap between the groups (see Figure 7). More clear distinctions between groups were seen with the 8 kHz tone pip (see Figure 8) with the ephrin-B3<sup>null</sup> group having the shortest conduction times and the ephrin-B3<sup>Lz</sup>



having the longest conduction times. For the 12 kHz tone pip (see Figure 9), the IWIs were shortest for the EphA4 group and the longest for the ephrin-B3<sup>Lz</sup>. No IWIs could be obtained for the ephrin-B3<sup>null</sup> in this case. It is interesting that for 8 kHz, the conduction times were the longest in the ephrin-B3<sup>Lz</sup> group, but the shortest when the stimulus was a 12 kHz tone pip.

### **Amplitude Measures**

Wave I amplitude at the loudest click decreased by 42% for the ephrin-B3<sup>Lz</sup> group, 60% for the ephrin-B3<sup>null</sup> group, and 49% for the EphA4 group. Amplitudes at the loudest 8 kHz tone pip showed decreases of 39% for the ephrin-B3<sup>Lz</sup> group, 55% for the ephrin-B3<sup>null</sup> group, and 37% for the EphA4 group for wave I amplitude. Finally, the loudest 12 kHz tone pip showed a decrease in wave I amplitude of 56% for the ephrin-B3<sup>Lz</sup> group, 81% for the ephrin-B3<sup>null</sup> group, and 63% for the EphA4 group. This revealed the most significant decreases in wave I amplitude to be for the ephrin-B3<sup>null</sup> group. The 2008 Miko et al. study, found the 54% smaller wave I amplitudes in the EphA4 group, as compared to the 49% smaller wave I amplitudes found in this study.

The amplitude of wave II for the click decreased by 78% for the ephrin-B3<sup>Lz</sup> group, 66% for the ephrin-B3<sup>null</sup> group, and 5% for the EphA4 group. The 8 kHz tone pip showed decreases of 73% for the ephrin-B3<sup>Lz</sup> group, 77% for the ephrin-B3<sup>null</sup> group, and 37% for the EphA4 group for wave I amplitude. Finally, the 12 kHz tone pip showed a decrease in wave II amplitude of 87% for the ephrin-B3<sup>Lz</sup> group, 81% for the ephrin-B3<sup>null</sup> group, and 48% for the EphA4 group. The Miko et al., (2008) study found the wave II amplitude for clicks to decrease by 56% in the EphA4 group, however, this study only found a 5% decrease in amplitude between the EphA4 group and the wild type group.

### **Other Miscellaneous Findings**

It is worth noting that the mutant mice all were significantly heavier than the wild type mice. This often required multiple injections of ketamine/xylazine in order to properly anesthetize the mouse for testing. Attempts were taken to control for age and this was done successfully without any age related effects in the analysis. This was important since some strains of mice have been shown to have early onset deterioration of hearing which results in higher auditory thresholds. Therefore, an accurate representation of hearing threshold was obtained in the mice before this aging process began. While attempts were made to test an equal number of male and female mice, this was not possible given the litters available at the time of testing. The results suggested that no gender differences existed, however, so analysis was not affected by that variable.

Finding no significant differences between the homozygous and heterozygous genotypes within each group was likely attributed to the small number of homozygotes. It was expected that homozygous mutants would have more severe auditory consequences and increases in thresholds, however the small number available for analysis may have prevented accurate statistics on this variable. This study was limited by the small number of subjects available at the time of testing. Future studies would benefit from an increase in sample size for homozygous and heterozygous mice to determine what, if any, differences exist. Of interest would be more homozygous ephrin-B3<sup>Lz</sup> and homozygous EphA4s

### **Future Research**

This study hoped to provide objective differences between the wild type mice and mutant groups in the latency of wave V. While the ABR measures were excellent at obtaining information about the early wave components, there was considerably more difficulty in identification of wave V and more variance in wave V among the wild type

mice and mutants. Therefore, some refinement of the protocol would be necessary if future studies were to look for group differences in the later wave components. Some possibilities to improve this based on our pilot study include optimizing the number of sweeps and filtering and trying to measure the negative potential following wave V (Moller & Jannetta, 1983). Direct assessment of the IC may be improved by measuring the binaural interaction component and the resulting evoked potential from sudden gaps in continuous white noise.

Experimenter biasing in peak selection could affect the external validity of the results as well. Future studies could have improved validity if unbiased volunteers were trained on selection of the peaks on randomized subjects and the volunteers participated in the analysis of the data. For this study, all peaks were selected by the same researcher.

Since this study succeeded in finding physiological differences in mutant mice, we may be able to apply this knowledge of normal and abnormal connections to design future experiments about the underlying mechanisms of appropriate functional development. Understanding which molecular signals are critical in development of auditory circuits is a crucial part about determining rational therapy approaches. Since effective treatment of hearing loss is dependent on an understanding of the normal auditory system development and organization, we could better plan treatment strategies for humans with early sensorineural hearing loss.

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