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Effect of EphA4 Signaling Mutations on Auditory Function

Michelle Rebekah Gerringer

A dissertation submitted to the Graduate Faculty of

JAMES MADISON UNIVERSITY

In

Partial Fulfillment of the Requirements

for the degree of

Doctor of Audiology

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The author would like to extend her sincere gratitude to the members of the James Madison University community who participated in this project. Thank you, specifically, to my advisor Dr. Lincoln Gray, who showed such patience, encouragement, and humility in the pursuit of knowledge. Your mantra, "perfection is elusive", became a challenge and an encouragement. Much gratitude to you as well for the countless cups of tea. Thank you to my committee members for their willingness to offer critical comments and to give openly of their time to offer guidance. Many thanks to undergraduates Allen Derina, Joe Balsamo, and Lauren Pitek, for the many hours spent collecting data, peak picking, and genotyping the mice. To her family and friends, all of your prayers and well-wishes meant so much. My greatest source of strength and guidance throughout this project came from Almighty God, who is a provider of peace to those who trust in Him. I am humbled by your interest in this work and in my life; you are all wonderful!

Introduction to the Reader

This dissertation is written in a manuscript format toward the intent of possible future publication of the work. Part I is the manuscript itself and is intentionally brief in reviewing other research in this topic area. An extended literature review is found in Part II. It provides greater detail about the premises of auditory research in murines with gene mutations as well as an overview of the current knowledge base regarding Eph/ephrin signaling in auditory development.

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Abstract

Neural pathways underlie the ability of the auditory system to perceive sound. Organization of neural pathways into functional auditory circuitry is accomplished in part by Eph and ephrin signaling proteins. One of these signaling proteins, the EphA4 receptor tyrosine kinase protein, acts as an axon-guidance molecule to aid in target selection and to maintain tonotopicity in the auditory brainstem and midbrain. Genetic mutations of the EphA4 protein have been shown to affect structural auditory development, but there is limited research which shows the functional effects of these mutations. The goal of the present study was to determine the functional effects of EphA4 *lacZ* mutations on auditory processing using physiologic measures. Auditory Brainstem Response (ABR) measures including summating potential (SP) amplitude were recorded in EphA4 *lacZ* mutant mice (with C57BL/6J background strain) prior to three months of age and compared to a control group of wild-type littermates. ABR wave latency and threshold analysis in heterozygous mice showed no significant differences from controls.

Comparison of homozygous mutant mice to wild-type controls showed significantly elevated (poorer) ABR thresholds in the homozygous group for 8 kHz tone-burst, 12 kHz tone-burst, and click stimuli. SP amplitudes were increased in the homozygous group suggesting mutation related changes to the auditory system. Deficits in auditory function seen in the homozygous mutant strain provide evidence that normal EphA4 expression is necessary for normal auditory function. Preserved function in the heterozygous mutants suggests that one allele is sufficient for normal function at

approximately one to three months of age. Our findings support the role of EphA4 in the development of the auditory function.

Part I: Manuscript

Chapter 1

Introduction

Eph/ephrin Signaling

Meaningful perception of sound relies on a rich network of neural pathways in the auditory system. Eph receptor tyrosine kinase proteins and their ligands, the ephrins, are known to be involved in the assembly of auditory circuitry. Both Ephs and ephrins are expressed broadly throughout the auditory brainstem and aid in early guidance of axons during development (Cramer & Gabriele, 2014). Ephs and ephrins are organized into classes A and B, with binding typically occurring within shared classes (e.g. ephrin-A ligands bind EphA receptors) (Cramer, 2005; Gale et al., 1996). Two exceptions to this class discrimination include the ability of ephrin-B ligands to bind EphA4 receptors (Gale et al, 1996) and the ability of ephrin-A5 to bind with EphB2 (Himanen et al., 2004).

The present study focused on EphA4, an individual protein of the Eph family, whose expression suggests its involvement in establishing tonotopically organized circuits in the auditory brainstem and midbrain (Gabriele et al., 2011). Current literature shows that EphA4 is expressed in nuclei throughout the auditory brainstem.

A study by Miko, Henkemeyer, and Cramer (2008), measured Auditory Brainstem Responses (ABR) in EphA4 mutant mice and found increased ABR thresholds, prolonged wave III latency, and increased amplitudes in both homozygous and heterozygous groups, suggesting that EphA4 is necessary for normal auditory function.

Murines in Auditory Research

The mouse serves as a valuable model for study of mammalian hearing due to its similarities to the human anatomy and genome. Approximately 99% of murine genes have homologous regions in humans (Avraham, 2003). The mouse model is ideal for experiments that use genetic manipulations to study the role of a gene or protein. In the current study, EphA4 strain *lacZ* mutants were compared to their wild-type littermates using physiological measures of auditory function to determine the role of EphA4 in the auditory system. This particular *lacZ* mutation includes a genetic manipulation in which the signaling portion of the gene which encodes the EphA4 protein is deleted, rendering it incapable of signaling. Any difference found between the mutant and wild-type groups would help to illuminate the role of EphA4 in auditory function.

Murines in the present study were bred on a C57BL/6J background strain. A large body of research exists using this murine strain and much is known about the normal functioning of C57BL/6J murines. C57BL/6J mice have rapid auditory development reaching functional onset of hearing at postnatal day 14 (Mikaelian & Ruben, 1965). Zheng, Johnson, and Erway (1999) recorded ABR using 8 kHz, 16 kHz, 32 kHz, and click stimuli and found C57BL/6J strain mice had normal ABR thresholds 33 weeks after birth. In the present study, all mice were tested by 91 days after birth using stimuli of similar frequencies. In general, murine hearing ranges from 0.5 kHz-120 kHz, with the greatest sensitivity from 12-24 kHz (Zheng et al., 1999). The current experiment utilized 8 kHz tone-burst, 12 kHz tone-burst, and click stimuli in order to test stimuli from the most sensitive frequency region in mice.

Auditory Brainstem Response

The ABR is a measure of neural synchrony which shows the function of the auditory brainstem up to the level of the inferior colliculus. While behavioral thresholds are the gold standard to measure hearing perception, the ABR can be used to predict audiometric thresholds in mice (Zheng et al., 1999). The ABR has also been widely used for genetic research, due to its ease of recording, reliability, and sensitivity.

ABR waveforms in mice are primarily composed of waves I-III with the largest amplitude in waves I and II (Miko et al., 2008). Waves IV and V often blend into the noise floor and are difficult to identify (Burkard, Don, & Eggermont, 2007; Zheng et al., 1999). On some waveforms, the summating potential (SP) is also visible. Both the SP and the ABR are neural responses which maintain a constant polarity, regardless of the polarity of the stimulus. When present, the SP appears as a shoulder on the leading edge of wave I (Sergeyenko, Lall, Liberman, & Kujawa, 2013). Analysis of the SP can be used as a gross measure of hair cell function in the frequency regions of the basilar membrane tested. Wave IV, wave V, and the SP were not observed in every waveform, thus only when those components were clearly present and replicable were they analyzed in the current experiment.

In examining the effect of EphA4 mutations on brainstem function, ABR measures can be a useful tool. However, it must be considered that production of the ABR relies on both peripheral and central auditory function. Increased (poorer) ABR thresholds could be a byproduct of mutation-based degradation to the cochlea and/or to the brainstem. SP amplitude measures were also collected in subjects of the current study as a gross measure of cochlear function. SP and ABR results were analyzed together to

determine if functional differences exist in mutant groups and to determine the site of dysfunction.

General Statement of the Problem

The EphA4 protein has been implicated in signaling the structural development of the auditory system, but there is minimal research, which shows the functional effects of these mutations. The goal of the present study was to determine the functional effects of an EphA4 *lacZ* mutation (which is incapable of signaling) on auditory processing using physiologic measures. The following hypotheses were addressed in this study:

(1) It was hypothesized that heterozygous EphA4 mutant mice would have slightly elevated (poorer) ABR thresholds and prolonged wave latencies compared to wild-type littermates, indicating an effect of a single allele mutation on the auditory system.

(2) It was expected that homozygous EphA4 mutant mice would have significantly elevated (poorer) ABR thresholds and prolonged wave latencies compared to wild-type littermates, indicating a null mutation causes more profound deficits in the auditory system.

(3) It was also hypothesized that the site of dysfunction in mutant mice would likely be in the auditory brainstem, based on wave latency, ABR threshold, and SP amplitudes, similar to results of Miko et al. (2008).

Chapter 2

Materials and Methods

Subjects

Mice with *lacZ* mutations to the EphA4 protein were bred on a C57BL/6J background strain, and housed in an animal housing facility at James Madison University. There were three mice in both experimental groups, EphA4$^{lacZ/+}$ (n=3) and EphA4$^{lacZ/lacZ}$ (n=3), and 12 wild-type littermates in the control group, EphA4$^{+/+}$ (n=12). All ABR measures were recorded prior to three months of age to prevent any concern of age-related hearing loss seen in C57BL/6J strain mice (Zheng et al., 1999). Genotyping was performed after testing, using polymerase chain reaction (PCR) of tail samples. Approval for this experiment was given by the James Madison University Institutional Animal Care and Use Committee (IACUC Protocol #A12-12).

Preparation

Mice were anesthetized with an Intramuscular (IM) injection of 150 mg/kg ketamine and 30 mg/kg xylazine. Mice received a 1/3 additional dose of anesthesia as needed during testing. Testing took place in a sound treated booth equipped with an infrared camera providing visual monitoring throughout recordings. Mice were positioned on an animal blanket control unit to preserve body temperature during testing. Subcutaneous needle electrodes, with impedances $\leq 1k\Omega$, were placed at the vertex (non-inverting), mastoid (inverting), and back (ground), and connected to the Tucker Davis RA4PA 4-channel Pre-amp. A Y-shaped closed tube delivery system was positioned into the external auditory meatus of one ear only. Stimuli were presented using a TDT EC1

high-frequency electrostatic speaker into one ‘arm’ of the Y-tube, and monitored with an Etymotic research ER-7C probe microphone in the other arm of the closed-tube system. An Agilent 35670A Dynamic Signal Analyzer was used to do real-time monitoring of peak frequency, amplitude, and bandwidth of the stimulus during ABR recordings.

Genotyping Procedures

Genotyping was performed after testing, using PCR amplification of DNA from tail samples. Light anesthesia (3% isofluorane) was administered to mice in order to obtain tail samples (~2 mm) and perform ear tagging for identification. Tail samples were placed in a tail denature buffer (25 mM NaOH, 0.2 μM EDTA) for 1 hour at 98°C. Tails were then neutralized with 40 mM Tris-HCl, pH 5.5 neutralization solution. JumpStart™ REDTaq® ReadyMix™ Reaction Mix (Sigma-Aldrich P0982) was used to perform PCR amplification (94°C 30 s; 31cycles: 94°C, 30 s, 61°C, 30 s, 72°C, 2 min; one final elongation at 72°C, 10 min) in conjunction with the following primer sequences: EphA4-forward 5’-AGACATTCCAGAAGAGGGAGTCAG-3’; EphA4-reverse 5’-ATAGACAGGACACAGTGAAGCCAC-3’; lacZ-forward 5’-GCACCGATCTAGTTGAAGACATC-3’; lacZ-reverse 5’-CACGCCATACAGTCCTCTTCACATC-3’. PCR products with EphA4-forward and EphA4-reverse primer set yielded a 376 base pair band (wild-type) on gel electrophoresis testing, while the *lacZ*-forward and *lacZ*-reverse primer set produced a 729 base pair band (mutant Eph-A4^{lacZ} allele).

Stimulus & Recording Procedures

Tucker-Davis hardware (System III) and BioSig software were used to generate 8 kHz tone-burst, 12 kHz tone-burst, and click stimuli. Stimuli were chosen at frequencies which would best target optimal regions of murine hearing (Ehret, 1983). Stimuli were presented at a rate of 39.1 clicks/sec at 90, 70, 60, 55, 50, 45, 40, 35, 30, 25, & 20 dB nominal SPL presentation levels (where dB nominal SPL is a reference to the expected intensity of the stimulus, which was later calibrated to exact level). Input was sampled at a rate of 25 kHz, digitally filtered between 300-3000Hz, and displayed on a 10 ms time window. Tone-burst stimuli were 5ms in duration and were shaped by a Blackman window with a 0.5 ms rise/fall time and a 4 ms plateau. Click stimuli were 0.1 ms in duration providing calibrated energy between 1-10 kHz. ABR recordings were measured in a one-channel recording of one ear only, and the order of stimuli presentation was randomized. Four 100 sweep replications, two condensation and two rarefaction, were taken at each intensity level.

Offline Analysis

ABR recordings were analyzed for threshold, wave latency, wave I and II amplitude, and SP amplitude. All ABR analyses were performed blind to genotype. Data from Miko et al. (2008) were used to estimate regions of expected ABR wave latencies in EphA4 mutant murine strains. Stimuli were calibrated from dB nominal SPL on the BioSig software to dB ppeSPL after completion of testing.

A MATLAB (R2013A) program presented two paired traces, each the sum of one condensation and one rarefaction trace, at decreasing intensities. Replicability of both

summed waveforms was observed at each presentation level. Threshold was defined as the lowest presentation level observed to elicit waveforms characteristic of those seen at higher intensities. Subjects with thresholds unable to be detected at 90 dB nominal SPL were defined to have a threshold of 95 dB ppeSPL.

Wave latencies (in ms, corrected for tube length) were estimated by observers looking at averages of all four recordings at all intensities above threshold. Wave I-V peaks were chosen when present, but often poor waveform morphology made later wave components impossible to detect. Amplitude (peak-valley) was calculated from peak of the wave to the following valley for waves I and II. Waves I and II were chosen for amplitude study in this experiment because they are the largest in murine ABRs (Miko et al., 2008).

SP amplitudes (baseline to peak) for the 90 dB nominal SPL recordings were obtained as a gross measure of cochlear hair cell function. Baseline was the average of points during pre-stimulus baseline ("negative time' after correction for tube length). SP amplitude was marked only when visible. The SP was not visible on all waveforms, making it difficult to analyze, especially in the poor morphology waveforms of the homozygous group. Due to absence of any response on ABR testing, one subject in the homozygous group was excluded in this measure.

Chapter 3

Results

Inter-Observer Reliability

A random subset of ABRs (265 of 415 total traces, or 64% randomly selected) was analyzed by two independent observers as a measure of inter-observer reliability. Correlation squared showed good agreement between observers for wave I latency (r^2=0.97), wave II latency (r^2=0.79), and for ABR thresholds (r^2=0.81). Figure 1 shows scatterplots of wave I and II latency from each observer. These robust correlations show strong inter-observer reliability for our data analysis of both thresholds and wave latency.

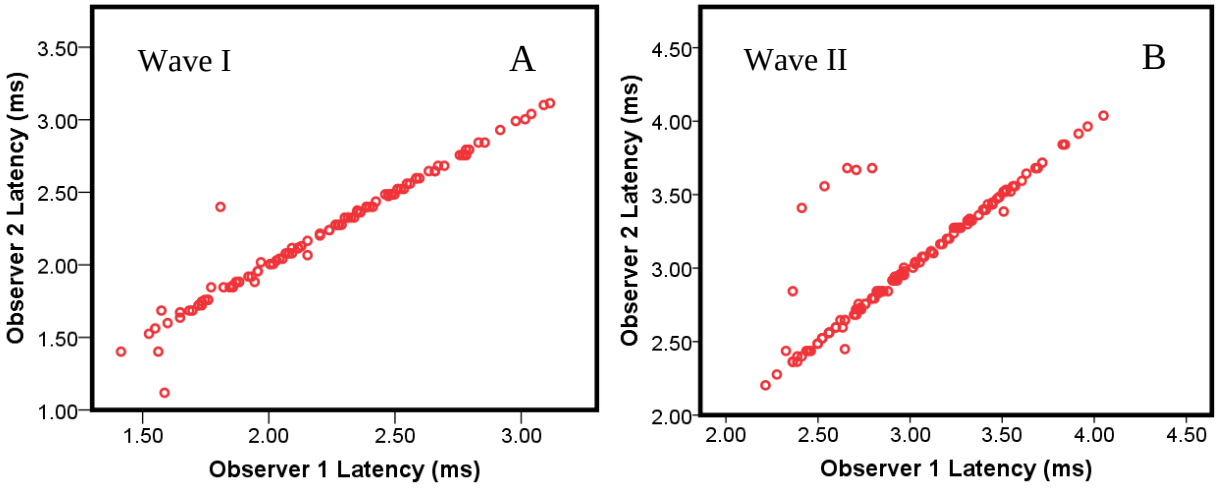


Figure 1. Scatterplots showing inter-observer reliability. Correlations squared of two observers' judgments of (A) Wave I latency (r^2=0.97) and (B) Wave II latency (r^2=0.79). Units are milliseconds (ms).

Auditory Brainstem Response

ABRs were recorded in EphA4 mutant mice and compared to their wild-type littermates. Group averaged ABR waveforms elicited by a click stimulus from wild-type, heterozygous, and homozygous groups are shown in Figure 2. Threshold estimates from these averaged waveforms are indicated by the red arrows. In wild-type and heterozygous groups, threshold estimates were the same and waveform morphology was similar. In the homozygous group, the threshold estimate was elevated and waveform morphology was very poor. Averaged waveforms shown in Figure 2 are representative of individual waveforms seen throughout the current study.

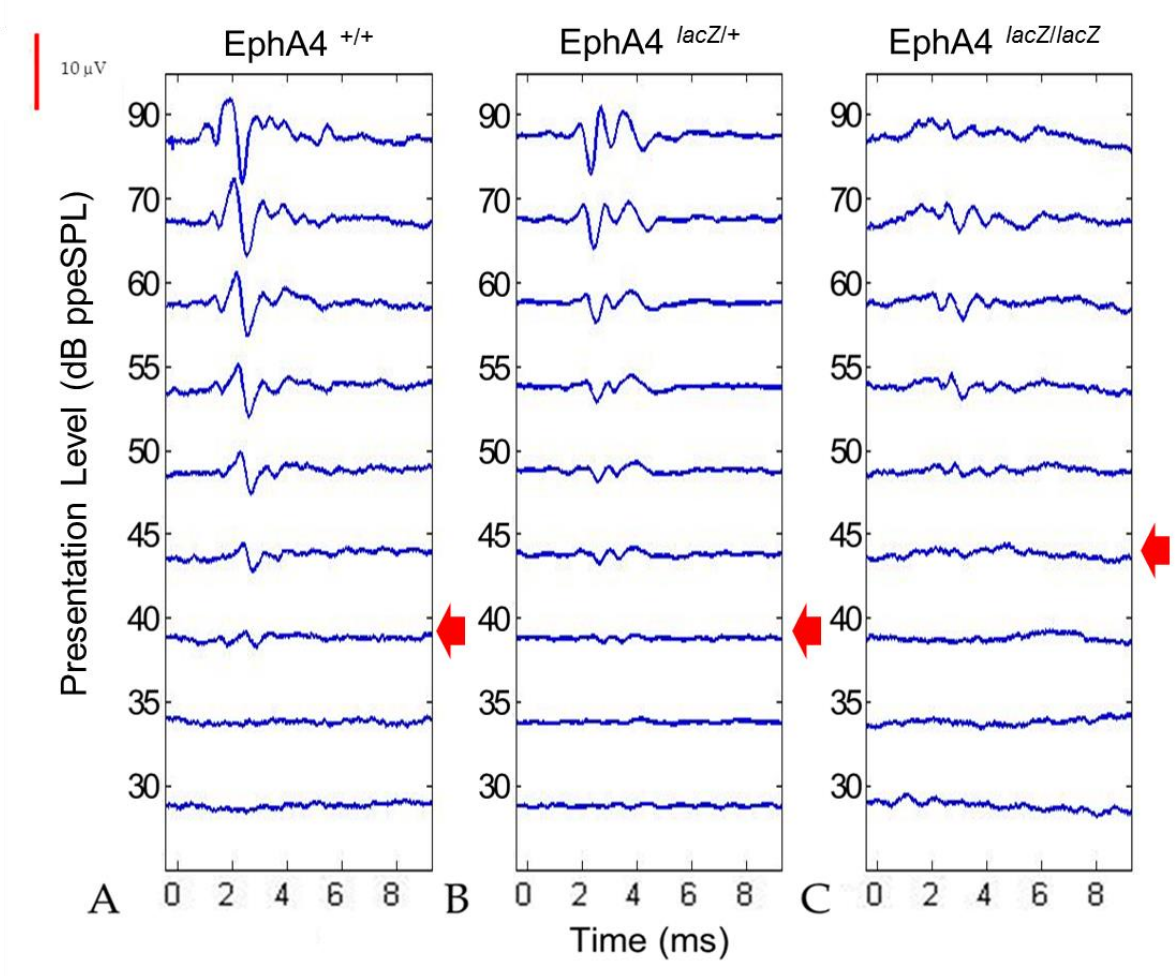


Figure 2. Group average ABR waveforms from (A) 11 EphA4$^{+/+}$ mice, (B) 3 EphA4$^{lacZ/+}$ mice, and (C) 3 EphA4$^{lacZ/lacZ}$ mice, elicited by a click stimulus and displayed across presentation levels. All averaged waveforms are shown using the same fixed amplitude scale. Red arrows indicate thresholds estimated from averaged traces.

ABR Thresholds

A repeated measures Analysis of Variance (ANOVA) was used to analyze ABR thresholds. The ANOVA included a between-subjects factor of mutation on three levels (EphA4$^{+/+}$, EphA4 $^{lacZ/+}$, and EphA4 $^{lacZ/lacZ}$) and a within-subjects factor of stimulus on three levels (click, 8 kHz tone-burst, and 12 kHz tone-burst). ANOVA of 18 subjects revealed a significant main effect of mutation on threshold ($F_{2, 14}$=3.88, p=0.046, pη2=0.357, or large effect size). Least significant difference Post Hoc tests showed heterozygous mice had no significant difference in thresholds when compared to wild-type littermates (p=0.670). Homozygous mutations of EphA4 yielded significantly elevated (poorer) thresholds when compared to wild-type (p=0.021) and heterozygous (p=0.030) littermates.

Figure 3 shows ABR thresholds for homozygous, heterozygous, and wild-type groups for all stimuli used in the study. A significant effect of stimulus on threshold was also found (p=0.017), as the click stimulus yielded lower (better) ABR thresholds than the tone-burst stimuli. Lower (better) click-ABR thresholds were likely due to increased neural synchrony often seen with broader basilar membrane stimulation and transient onset. ABR thresholds in homozygous EphA4 mutants were significantly elevated (poorer) compared to ABR thresholds in the heterozygous or wild-type groups, suggesting the EphA4 protein is essential for normal auditory function.

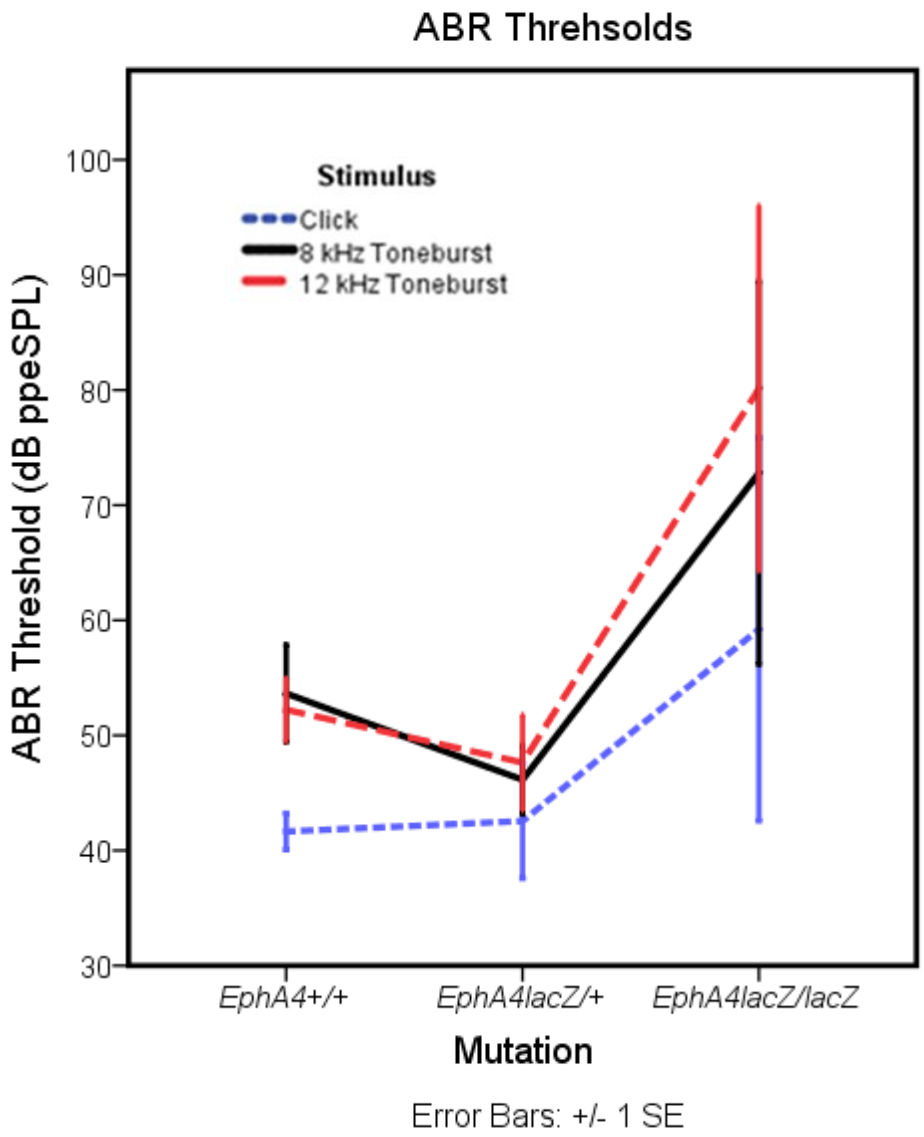


Figure 3. Line graph of ABR thresholds for click, 8 kHz tone-burst, and 12 kHz tone-burst stimuli. ABR thresholds were significantly elevated (poorer) for EphA4$^{lacZ/lacZ}$ mice.

Wave I Latency

Latency-intensity functions were analyzed to search for any interactions between latency of wave I and the intensity of the stimulus in mutant groups. An Analysis of Covariance (ANCOVA) was used to evaluate the effects of presentation level, mutation, and stimulus type on wave I latency. The ANCOVA included a covariate of presentation level on twelve levels (95, 90, 70, 60, 55, 50, 45, 40, 35, 30, 25, & 20 dB ppeSPL), a between-subjects factor of mutation on three levels (EphA4$^{+/+}$, EphA4 $^{lacZ/+}$, and EphA4 $^{lacZ/lacZ}$), and a between-subjects factor of stimulus on three levels (click, 8 kHz tone-burst, and 12 kHz tone-burst). As expected, there was a highly significant effect of presentation level ($F_{1, 263}$ =50, p<0.001, $_p\eta^2$ = 0.161, or large effect size) and there was no main effect or interaction involving either stimulus or mutation (p>0.050). As the presentation level of the stimulus decreased, latency of wave I was increased for all groups, and no significant differences were found in these trends between wild-type, heterozygous, or homozygous groups.

All Wave Latencies

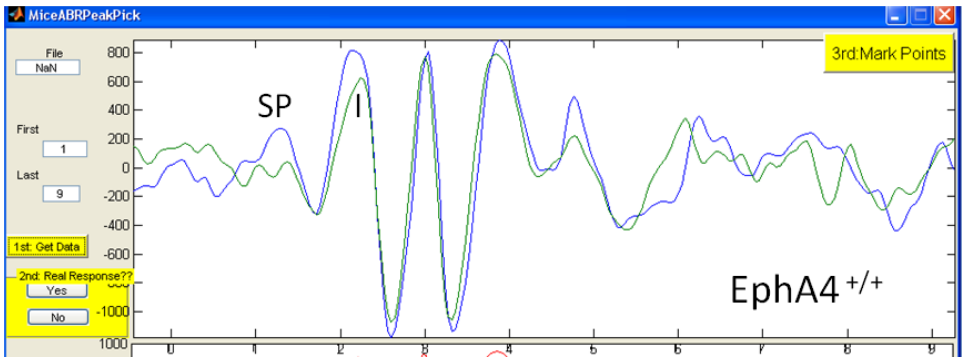
Examination of absolute ABR wave latencies from the 90 dB ppeSPL presentation level revealed no significant effect of mutation on absolute wave latencies. An ANCOVA was used to evaluate effects of wave, mutation and stimulus type on wave latencies. The ANCOVA included a covariate of wave on five levels (I, II, III, IV, V), a between-subjects factor of mutation on three levels (EphA4$^{+/+}$, EphA4 $^{lacZ/+}$, and EphA4 $^{lacZ/lacZ}$), and a between-subjects factor of stimulus on three levels (click, 8 kHz tone-burst, and 12 kHz tone-burst). As expected, there was a highly significant effect of wave ($F_{1, 195}$ =955.8, p<0.001) and there was no main effect or interaction involving either

stimulus or mutation (p>0.050). Absolute wave latencies were similar across wild-type, homozygous, and heterozygous groups. See the appendix for figures of both mean wave latency and latency-intensity functions.

Summating Potential: A Gross Estimate of Cochlear Function

ABR measures rely on proper transmission of sound through the external ear, middle ear, and cochlea. In order to make conclusions regarding the function of the auditory brainstem in mutant groups, it must be determined how the EphA4 mutations affect the middle ear or cochlea. In the present study, SP amplitudes were measured from all ABR recordings where present. The SP is generated by cochlear hair cells (Dallos, Schoeny, & Cheatham, 1972). Comparison of SP amplitudes across groups can provide a gross estimate of cochlear function. Figure 4 shows representative ABR waveforms, elicited by an 8 kHz tone-burst stimulus, from wild-type, heterozygous, and homozygous groups with SP and wave I marked.

A



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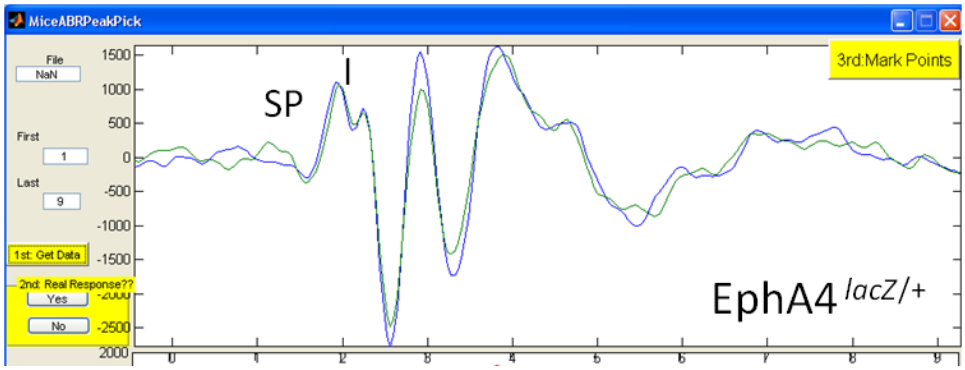

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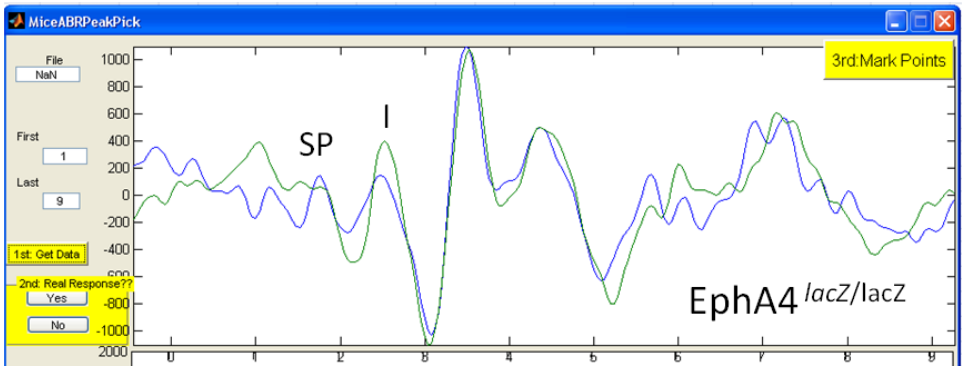


Figure 4. Representative ABR waveforms elicited by a 8 kHz tone-burst stimulus from an (A) EphA4$^{+/+}$ mouse, (B) EphA4$^{lacZ/+}$ mouse, (C) EphA4$^{lacZ/lacZ}$ mouse. SP and wave I or action potential (AP) for the 90 dB nominal presentation level are marked on each waveform. Vertical scales are different for each waveform.

SP amplitude was generally larger in the homozygous group when compared to wild-type littermates, even though absolute thresholds were poorer (Figure 5). A repeated measures ANOVA was performed to determine the effect of mutation on SP amplitude. The repeated measures ANOVA included a within-subjects factor of stimulus on three levels (click, 8 kHz tone-burst, and 12 kHz tone-burst) and a between-subjects factor of mutation on three levels (EphA4$^{+/+}$, EphA4 $^{lacZ/+}$, and EphA4 $^{lacZ/lacZ}$). Results showed a highly significant effect of mutation ($F_{2, 9}$ =550, p<0.001, $_p\eta^2$=.99, or large effect size), with no multivariate effect of stimulus (p=0.100) nor stimulus-by-mutation interaction (p=0.280). Poor morphology in homozygous waveforms made choosing SP impossible in many cases. In the present study, only one of three tested homozygous mouse had an identifiable SP in every stimulus condition. As a result, least significant differences Post Hoc tests for SP amplitude were not able to be analyzed. Wild-type and heterozygous mice had smaller SP amplitudes than homozygous mice. Elevated SP amplitude in the homozygous group may suggest mutation related changes to the auditory system.

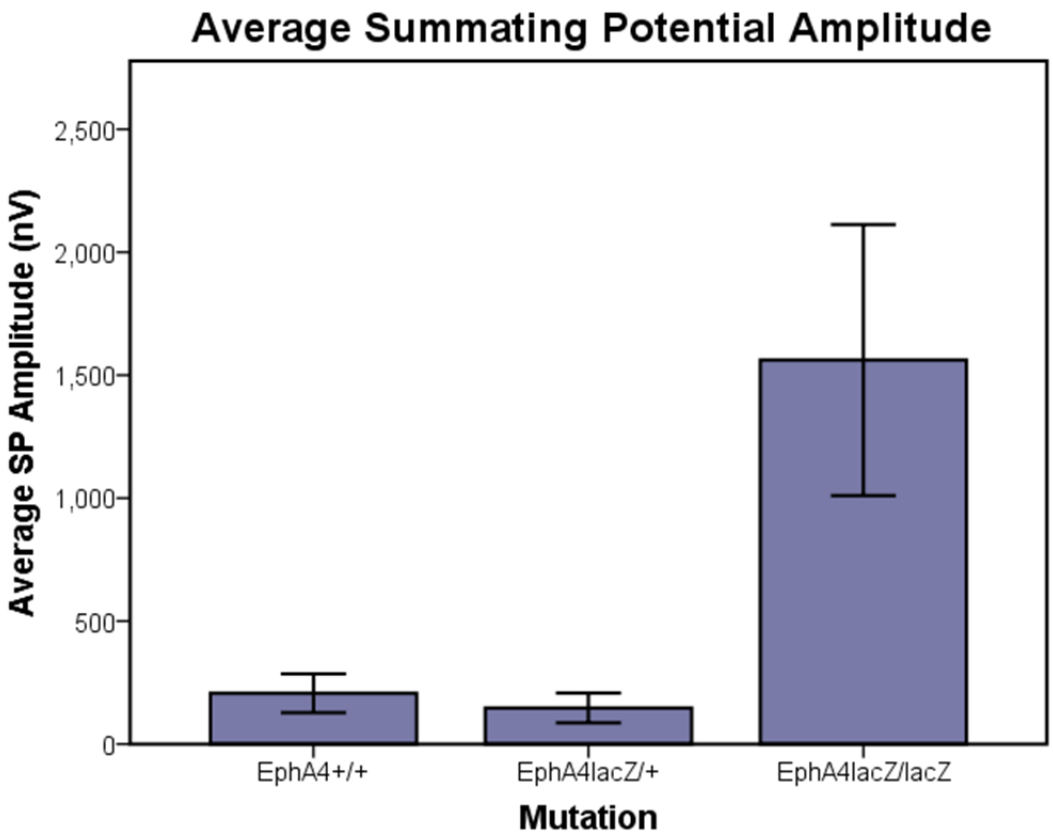


Figure 5. Bar graph of average SP amplitude from 8 kHz, 12 kHz, and click stimuli. Amplitudes in the homozygous group were significantly elevated ($p < 0.050$).

Chapter 4

Discussion

Mutations of the EphA4 protein have been shown to affect structural development of the auditory system, but there is minimal research showing the functional effects of these signaling proteins. The goal of the current study was to determine the functional effects of EphA4 *lacZ* mutations on the auditory system using physiological measures. The results of this experiment showed that the EphA4 protein is necessary for normal ABR thresholds, and that one normal allele is sufficient for normal ABRs.

Auditory Brainstem Response Thresholds

ABR thresholds in the heterozygous, EphA4 $^{lacZ/+}$ group, showed no statistical difference from their wild-type littermates, EphA4 $^{+/+}$. Our results suggested that a single normal allele of the EphA4 gene is sufficient for normal ABRs. According to Gale et al. (1996), both ephrin-A and ephrin-B ligands are able to bind to the EphA4 receptor. This double-binding to EphA4 might make it possible, perhaps, for a heterozygous EphA4 mutant with only one working allele to have sufficient signaling for normal development. These results differ from Miko et al. (2008), which is the only other known study of EphA4 mutant murine ABRs. Miko et al. (2008) found that heterozygous and homozygous (EphA4 $^{lacZ/lacZ}$) mice showed elevated ABR thresholds compared to the wild-type group, with poorer thresholds in the homozygous group. In their experiment, Miko et al. used mice with null mutations of EphA4, while the present study utilized *lacZ* mutations. It is possible that this slight difference in the mutation contributed to the difference in results found for the heterozygous mice. Another possible explanation for

this difference in ABR threshold in the heterozygous group is age of subject at the time of testing. In their experiment, Miko et al. (2008) tested mice from postnatal day 18-20, while mice in the present study were tested from postnatal day 43-91. If heterozygous mice mature more slowly or adapt over time, having only one normal allele, this could explain the discrepancy between ABR thresholds in the present study and in Miko et al. (2008).

Miko et al. (2008) suggested that deficits caused by the Eph receptor may be ameliorated as the mouse ages. Findings in the present study, where mice were tested later in development yet prior to onset of age-related hearing loss, would suggest that as the mouse ages, some compensation may occur in heterozygous mice. While mice tested from postnatal days 18-20 in the Miko et al. (2008) study had significantly elevated ABR thresholds, mice in the present study, tested from postnatal day 43-91, had similar ABR thresholds to the wild-type group. Homozygous mice with two abnormal alleles had elevated ABR thresholds and were unable to compensate for the mutation.

ABR thresholds in the homozygous group were significantly elevated (poorer) compared to the wild-type or heterozygous groups. Elevated thresholds in the homozygous group were seen across all three stimuli tested, similar to the Miko et al. (2008) study. While heterozygous mice, with a single-allele mutation, maintained normal ABRs, homozygous mice, with a bi-allele mutation, showed significant impairment of the ABR. Waves I and II have the largest wave amplitudes in murine ABRs (Henry, 1979), and are therefore the most likely to be present at low intensity levels when searching for threshold. Primary generators for waves I and II are found at the auditory nerve and cochlear nucleus, respectively (Moller & Janetta, 1985). The auditory nerve and cochlear

nucleus were, therefore, highly likely to be affected by mutations of EphA4. Elevated (poorer) ABR thresholds in the homozygous group suggest that the mutation has an effect on the ability of the auditory nerve to respond to stimuli of intensity normally sufficient for a response.

Summating Potential: Possible Effect of EphA4 Mutation on Auditory System

SP amplitudes in both wild-type and heterozygous groups were comparable, while SP amplitudes in the homozygous group were significantly elevated. Cochlear hair cells in homozygous mutants were apparently able to produce a very robust SP, despite elevated (poorer) ABR thresholds. OAEs are also generated by cochlear hair cells and could be used as another tool to examine cochlear hair cell function. Currently, there is no published research reporting OAEs in EphA4 mutant mice, but Howard et al. (2003) found that mutation of EphB1 and EphB3 led to diminished distortion product otoacoustic emissions (DPOAEs) in mutant mice as compared to wild-type littermates, indicating EphB1 and EphB3 can have some effect on cochlear function. As EphA4 is expressed in the cochlea and other Ephs are known to diminish cochlear function, it is possible that elevated SP amplitudes and poor ABR thresholds could be a result of dysfunction in the cochlea. In contrast, Miko et al. (2008) observed prolonged latencies of wave III in EphA4 homozygous mutant mice and suggested this may reflect abnormality of the superior olivary complex. Results from Miko et al. (2008) suggest dysfunction in the brainstem in EphA4 mutant mice, with elevated (poorer) ABR thresholds in these mice. Without DPOAE measures or significant wave latency differences, it is difficult to identify the sites of dysfunction in these mice.

Change to efferent system function, may be another possible explanation for elevated ABR threshold and SP amplitude in homozygous mice. The efferent feedback system may be compensating for central deficits in the homozygous mutants by increasing the cochlear amplifier. Previous studies have shown that when the MOC is activated, the cochlear microphonic increases in amplitude while the action potential decreases in amplitude (Gifford & Guinan, 1987). SP amplitude increases may reflect similar changes in the efferent system. Thus, our data implicate a possible effect of homozygous mutation on efferent activity, auditory nerve, and/or the cochlea. More in-depth study is needed to determine the exact nature of these relationships.

Future Research

The current study used the SP, as a gross estimate of cochlear hair cell function, in tandem with the ABR, a measure of auditory brainstem function. We found that homozygous mice had both elevated (poorer) ABR thresholds and elevated (better) SP amplitudes. These SP recordings were based on an unconventional testing procedure using transdermal electrodes to measure SP amplitude rather than traditional electrocochleography, with electrodes positioned much closer to the cochlea. Results of the SP amplitude comparisons should be interpreted with caution, as amplitude measures are historically less reliable than other measures. Future studies should attempt to determine the site of dysfunction in EphA4 mutant mice by recording DPOAE in conjunction with ABR. Elevated SP amplitudes in the homozygous group may also suggest an effect of EphA4 mutation on the efferent system. Future research should

focus on testing the efferent system directly with DPOAE and/or compound action potential suppression measures.

Conclusions

EphA4 proteins are necessary for normal auditory function. ABR testing in the present study revealed that homozygous but not heterozygous EphA4 gene mutations reduce ABRs significantly. The role of EphA4 in the formation of the auditory system is, therefore, functional as well as structural. Significantly enlarged SP amplitudes in homozygous mutants may suggest changes in the efferent system, cochlea, and/or auditory nerve in these mutants. Further research should focus on illuminating the role of EphA4 in the cochlear and efferent system using DPOAE suppression measures.

(1) Heterozygous EphA4 mutant mice did not have significantly elevated (poorer) ABR thresholds or prolonged wave latencies compared to wild-type littermates. Thus, a single normal allele of the EphA4 gene is sufficient for normal ABRs.

(2) Homozygous EphA4 mutant mice had significantly elevated (poorer) ABR thresholds, but did not have prolonged wave latencies compared to wild-type littermates. Thus, a bi-allele mutation of the EphA4 gene results in significantly altered auditory function.

(3) Changes in EphA4 mutant mice may reflect dysfunction in the efferent system, cochlea, and/or auditory nerve.

Part II: Expanded Literature Review

Introduction

Since the formal manuscript above is a terse description of a study of mice and how EphA4 protein mutations affect their ABRs, this literature review will expand upon four general topics: (1) murine auditory system, (2) EphA4 gene mutations, (3) ABRs in mice, and (4) goals of the current study. The first section on the murine auditory system includes a review of the peripheral and central auditory systems and of C57BL/6J strain mice. The second section discusses Eph/ephrin signaling with special focus on EphA4 expression in the auditory system. The third section defines the ABR and reviews past studies of ABRs in mice. The fourth and final section discusses the goals of the current study and our proposed hypotheses.

Murine Auditory System

Development

A thorough review of the murine auditory system must consider development in both the afferent and efferent pathways. Development in the afferent auditory system reaches normal adult structure and function at postnatal day 10-14 (Mikaelian & Ruben, 1965). In the cochlea, growth of the organ of Corti continues until postnatal day 8-10 before reaching normal adult size. The adult murine cochlea is made up of two turns. Maturity of recorded cochlear potentials is reached by postnatal day 14 (Mikaelian & Ruben, 1965). Beyond the level of the cochlea, ABRs primarily reflect activity from the auditory nerve, cochlear nucleus, and superior olivary complex. Maturity of ABR measures is reached by postnatal day 18 for threshold and postnatal day 36 for wave

amplitude (Song, McGee, & Walsh, 2006). In order to ensure developmental maturity in all test subjects, mice in the present study were not tested prior to postnatal day 40.

Auditory Periphery

The mature frequency range of murine hearing spans from 0.5 to 120 kHz, with greatest sensitivity from 12-24 kHz (Ehret, 1983). Behavioral studies of adult murine hearing show similar areas of optimal sensitivity from 8-24 kHz (Radziwon, June, Stolzberg, Xu-Friedman, Salvi, & Dent, 2009). Based on these optimal frequency regions reported in the literature and on the limitations of our equipment, ABRs in the current study were conducted using 8 kHz tone-burst, 12 kHz tone-burst, and click stimuli.

Efferent System

Auditory development in the murine efferent auditory system differs widely from the development of the afferent system. Lateral olivocochlear (LOC) efferents projecting to the ipsilateral cochlea are larger in number than contralateral projections and have a uniform precise tonotopic distribution of fibers in cats. Medial efferent projections from the MOC to the contralateral cochlea are denser than ipsilateral projections in cats (Sahley, Nodar, & Musiek, 1997). OHCs show innervation from MOC fibers, while efferent terminals on the IHCs are supplied by LOC fibers (Maison, Adams, & Liberman, 2003).

The efferent auditory system in murines shows many similarities to other mammalian species. In both cats and mice, the distribution of MOC fibers shows the greatest innervation at the upper portion of the basal turn of the cochlea (Liberman, Liberman, & Maison, 2014; Maison, Adams, & Liberman, 2003; Sahley et al., 1997)

MOC activation in mice leads to inhibition of the OHC response and a decrease in the gain of the cochlear amplifier (Guinan, 2006). MOC inhibition of OHCs in mice has also been found to be most robust in the 16-22 kHz frequency region where the MOC innervation is the greatest (Liberman, Liberman, & Maison, 2014). Due to the thin and unmyelinated nature of LOC fibers, little is known about the effects of LOC activation (Guinan, 2006). The magnitude of MOC effects has often been examined with DPOAE's by measuring the amount of suppression of the cochlear amplifier. In quiet, MOC fibers must be activated invasively by shocks to the olivocochlear bundle, but are activated naturally in noise (Guinan, 2006).

Descending efferent pathways from the inferior colliculus may be involved in modulating cochlear processing. Descending projections from the inferior colliculus run primarily to the ipsilateral MOC fibers. The majority of MOC fibers then cross to the OHCs of the contralateral cochlea (Johnson, 2005). These descending projections may provide a pathway for input from the inferior colliculus to modify efferent suppression at the level of the cochlea. A study by Gifford and Guinan (1987) used purposeful stimulation of both the fourth ventricle olivocochlear bundle and MOC fibers to determine the effects of efferent stimulation. Following direct efferent stimulation, the auditory nerve compound action potential was decreased, while the cochlear microphonic was increased (Gifford & Guinan, 1987). This study would suggest that increased efferent activation may result in increased hair cell activity in the cochlea.

C57BL/6J Strain Murines

Murines in the present study were bred on a C57BL/6J background strain. A large body of research exists using this murine strain and much is known about the normal functioning of C57BL/6J murines. C57BL/6J mice have rapid auditory development, reaching functional onset of hearing at postnatal day 14 (Mikaelian & Ruben, 1965). In a study by Zheng et al. (1999), ABR thresholds were obtained in C57BL/6J mice to 8 kHz, 16 kHz, and 32 kHz tone-bursts and click stimuli. Normal ABR thresholds for adult C57BL/6J mice at 33 weeks are 39 ± 4, 33 ± 6, and 17 ± 3 dB SPL for click, 8 kHz tone-burst, and 16 kHz tone-burst stimuli, respectively. These are comparable to grand mean ABR thresholds calculated across 60 different inbred murine strains: click (38± 2.7), 8 kHz tone-burst (29± 3.4), and 16 kHz tone-burst (18± 4.2) in dB SPL (Zheng et al., 1999). By 100 weeks, these same C57BL/6J mice had thresholds which were 60 dB above normal means (Zheng et al., 1999). Normal behavioral thresholds in C57BL/6J mice age 30-60 days have been recorded at 35 dB SPL for an 8 kHz tone-burst stimulus (Miko, Nakamura, Henkemeyer, & Cramer, 2007). Age-related hearing loss in C57BL/6J strain murines has been well documented, thus making this strain an ideal research model for presbycusis (Parham, 1997; Zheng et al., 1999; Zhu et al., 2007).

Effects of aging on the auditory system also occur in the efferent system. In humans and CBA mice, studies have shown that efferent system function, determined by DPOAE adaptation, declines prior to the periphery (Jacobson, Kim, Romney, Zhu, &Frisina, 2003; Sun and Kim, 1999). A study by Sun and Kim (1999) measured efferent regulated DPOAE adaptation in both CBA/JNia and C57BL/6JNia mice at 2, 10, and 12

months of age. Results showed smaller DPOAE adaptation amplitudes in C57 mice at 2 months of age. This may suggest some changing aspects of the C57 efferent system by 2 months of age. Similar results were found in a study by Zhu et al. (2007) which measured contralateral suppression of DPOAEs in mice across the lifespan; MOC function of C57s was shown to be intact at 6 weeks, but began to decline rapidly, with changes in mid and high frequencies by 8 weeks.

EphA4 Gene Mutations

Gene Mutations

The mouse model is valuable for genetic research, as the genetic makeup of the murine auditory system is largely homologous with humans (Kikkawa et al., 2012). There are many well-defined mutant murine strains which are used in genetic research. The goal of using a gene manipulation in studying the auditory system is to compare a normal-functioning system with one that has a specific gene mutation.

In the current study, EphA4 strain *lacZ* mutants were compared to their wild-type littermates using physiological measures of auditory function to determine the effects of mutation to the EphA4 protein. This particular *lacZ* mutation includes a genetic manipulation in which the signaling portion of the gene encoding the EphA4 protein is deleted, rendering it incapable of signaling. Differences found between the mutant and control groups can illuminate the role of the protein in auditory function.

Eph/ephrin Signaling

Eph/ephrin family proteins are known to be involved in the development of the auditory system. Using cell-to-cell interactions (Davis et al., 1994), Ephs and ephrins

communicate information which is useful for axon guidance and target selection during development (Howard et al., 2003). Ephs are receptor tyrosine kinase proteins, which are tied to the membranous cell surface by way of a glycophosphatidylinositol (GPI) linkage (Cramer, 2005; Cramer & Gabriele, 2014). These receptor proteins have patterns of affinity to bind only with specific ephrin ligands. Binding can only occur after an ephrin ligand makes contact with specific Eph receptors which hold a mutual affinity. Stimulation of the receptor will cause a cascade of intracellular activity. Once a bond is formed, Eph and ephrin proteins are able to communicate bi-directionally via forward (ephrin-to-Eph) and reverse signaling (Eph-to-ephrin). Bidirectional signaling provides a channel for cell-to-cell communication, allowing for increased diversity of Eph function in development (Cramer, 2005).

Ephs and ephrins are divided into classes A and B. Typically, binding between Ephs and ephrins is exclusive to shared classes (eg. ephrin-A ligands bind EphA receptors) (Cramer, 2005; Gale et al., 1996). Within classes, there also exist greater affinities of particular Ephs to bond to particular ephrins (Cramer, 2005). Two exceptions to this class discrimination include the ability of ephrin-B ligands to bind EphA4 receptors (Gale et al., 1996) and the ability of ephrin-A5 to bind EphB2 (Himanen et al., 2004).

Elsewhere in the nervous system, Ephs are important in the formation of topographic maps (Cramer, 2005). In the cochlea, Eph proteins guide orderly patterning in the development of spiral ganglion neurons (Bianchi & Gray, 2002) and may aid in regulating ion concentrations inside the cochlea (Dravis et al., 2007).

EphA4 in the Auditory System

Expression of Eph receptors is seen throughout the auditory system in neural and non-neural regions. The present study focuses on the EphA4 receptor tyrosine kinase within the auditory system. Studies using immunohistochemistry found that expression of EphA4 was present in auditory and vestibular neurons, vestibular hair cells, and supporting cells in adult gerbils (Bianchi & Liu, 1999). Expression of EphA4 in mice is visible in the spiral ligament, in cells which develop into the osseous spiral lamina, and in regions surrounding the auditory nerve in adult mice (van Heumen, Claxton, & Pickles, 2000). In the auditory brainstem, EphA4 is expressed in the dorsal cochlear nucleus(DCN), the medial nucleus of the trapezoid body (MNTB), the lateral superior olivary complex (LSO), the dorsal nucleus of the lateral lemniscus, and in the inferior colliculus (Gabriele et al., 2011; Miko et al., 2007).

Current literature shows EphA4 has structural effects on auditory development. EphA4 is expressed throughout the auditory brainstem from the cochlear nucleus (Miko et al., 2007) to the inferior colliculus (Gabriele et al., 2011). Recent research by Gabriele et al. (2011) demonstrates the importance of Eph/ephrin cell-to-cell communication in the inferior colliculus. This experiment used DiI-labeling to illustrate lateral superior olive to inferior colliculus projecting in EphA4 and ephrin-B2 mutants, relative to controls. Discrete projection patterns were observed in the lateral cortex, while continuous, tonotopic layers were observed in the central nucleus of the inferior colliculus (LCIC, CNIC) during the first postnatal week. The development of these projection patterns correlates with discrete and graded EphA4 and ephrin-B2 LCIC and CNIC expression

patterns. With hearing onset, the expression of these signaling proteins is markedly down-regulated. Fluorescent tract-tracing in wild-type and mutant animals shows tonotopic organization in EphA4 heterozygous but not ephrin-B2 homozygous mutants (Figure 6) (Gabriele et al., 2011).

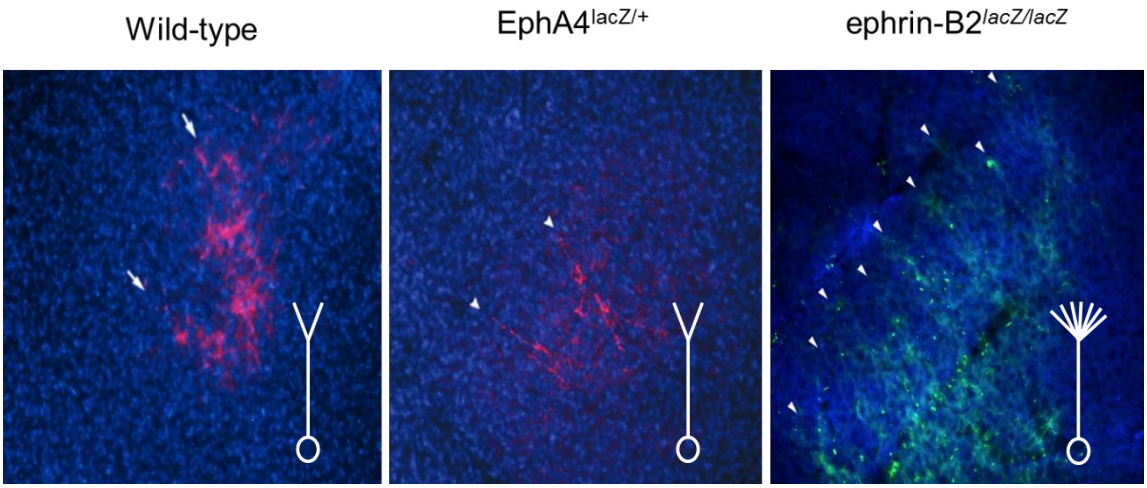


Figure 6. Lateral superior olive (LSO) projections to the inferior colliculus in normal and mutant mice. Small injections of an anterograde-transported dye were made in the LSO. Shown are the terminal projections in the right central nucleus of the inferior colliculus (CNIC). Arrows highlight characteristic tonotopic afferent projections in the inferior colliculus from LSO (images by Matt Wallace and Mark Gabriele). We expect the EphA4 homozygous group to show abnormal neural connections, similar to that seen in the related mutation on the far right. Dorsal is up and medial is to the left.

With widespread expression of EphA4 observed throughout the auditory system, the function of the auditory system must be examined across auditory structures. A study by Miko et al. (2008) recorded ABRs in EphA4 mutant mice to determine if the EphA4 protein is essential for normal function in the auditory brainstem. The results of this study showed delayed wave III latency, 54% decrease in peak I amplitude, and a 56% decrease in peak II amplitude. There was also a 75% increase in threshold, from 31.5 ± 1.26 dB SPL in EphA4+/+ controls to 55.0 ± 2.98 dB SPL in EphA4$^{-/-}$ knockout mice.

These data suggest EphA4 plays a role in maintaining normal auditory function.

Representative ABR tracing from Miko et al. (2008) can be seen in Figure 7.

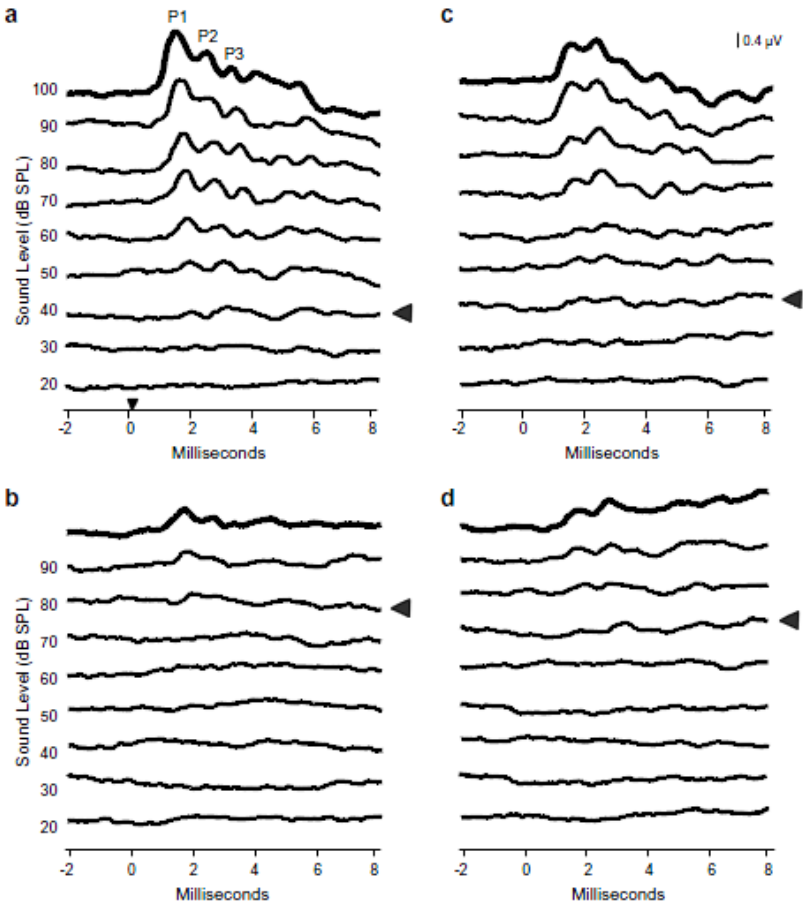


Figure 7. Representative ABR waveforms of EphA4 & ephrin-B2 wild-type and mutant mice from Miko et al. (2008). Waveforms show one subject from each of four groups (a) EphA4$^{+/+}$ (b), EphA4$^{-/-}$ (c), ephrin-B2$^{+/+}$ (d), and ephrin-B2$^{lacZ/+}$. Large black arrowheads indicate ABR threshold. The EphA4$^{+/+}$ mouse shown has a threshold near 40 dB SPL, while the EPhA4$^{-/-}$ has a much higher threshold, close to 80 dB SPL in this case.

Currently, there is no published research showing the effect of EphA4 mutations on DPOAE measures. One study, which performed DPOAEs on mice with knockout gene mutations to EphB1 and EphB3 receptors, found significantly reduced amplitudes in homozygous groups when compared to wild-type littermates. Therefore, they concluded that EphB1 and EphB3 are necessary for regulating cochlear OHC function (Howard et al., 2003).

Auditory Brainstem Response in Mice

The ABR is a measure of neural synchrony which yields functional information up to the level of the inferior colliculus. While behavioral thresholds are the gold standard to measure hearing perception, the ABR can be used to predict audiometric thresholds in mice (Zheng et al., 1999). ABR thresholds are typically higher than behavioral thresholds in murines. The ABR has also been widely used for genetic research, due to its ease of recording, general reliability, and sensitivity (Zheng et al, 1999).

The ABR is comprised of four to five waves and is generated by several nuclei along the auditory brainstem (Miko et al., 2008; Song et al., 2006). Due to the interconnected nature of the auditory brainstem and the nearness of these generators, each wave is generated by a primary generator as well as from other auditory nuclei. The primary generators for waves I, II, III, IV, and V in mice are found at the AP of the auditory nerve, cochlear nucleus, superior olivary complex, lateral lemniscus, and inferior colliculus, respectively (Henry, 1979; Moller & Janetta, 1985). ABR waveforms are primarily composed of waves I-III, with the largest amplitude in waves I and II (Miko et

al., 2008). Waves IV and V often blend into the noise floor and are difficult to identify (Burkard et al., 2007; Zheng et al., 1999).

Wave Generators by Species

Wave	Humans (Hall, 2007)	Mice (Moller & Janetta, 1985; Henry, 1979)
I	Distal Auditory Nerve	Auditory Nerve
II	Proximal Auditory Nerve	Cochlear Nucleus
III	Cochlear Nucleus& Superior Olivary Complex	Superior Olivary Complex
IV	Superior Olivary Complex	Lateral Lemniscus
V	Lateral Lemniscus & Inferior Colliculus	Inferior Colliculus

Table 1. Primary generators for each wave of ABR in humans and mice.

Protocols for choosing wave latency, threshold, and SP latency in mice differ slightly from protocols used in humans. Convention in murine ABRs is to choose the most positive voltage of a wave as its peak (Miko et al., 2008; Sergeyenko et al., 2013). Due to the frequent absence of waves IV and V, only the waves which are clearly replicable and present should be chosen. ABR thresholds in mice are often defined as the lowest sound intensity capable of producing a waveform representative of that seen at higher intensities (Polley, Cobos, Merzenich, & Rubenstein, 2006). On some waveforms,

the SP is also visible. Both the SP and the ABR are neural responses which maintain a constant polarity, regardless of the polarity of the stimulus. When present, the SP appears as a shoulder on the leading edge of wave I peak (Sergeyenko et al., 2013). Analysis of the SP can be used as a gross measure of hair cell function in the frequency regions of the basilar membrane tested.

Goals of the Current Study

Mutations of the EphA4 protein have been shown to affect structural development of the auditory system, but there is limited research showing the functional effects of these mutations. The goal of the present study was to determine the functional effects of EphA4 *lacZ* mutations on auditory processing using physiologic measures. We expected to see significant delay in wave latency and an increase in ABR threshold in experimental groups, with greater deficits in the homozygous group similar to the effects seen by Miko et al. (2008).

Appendix

Figures & Tables Not Included in Results Section

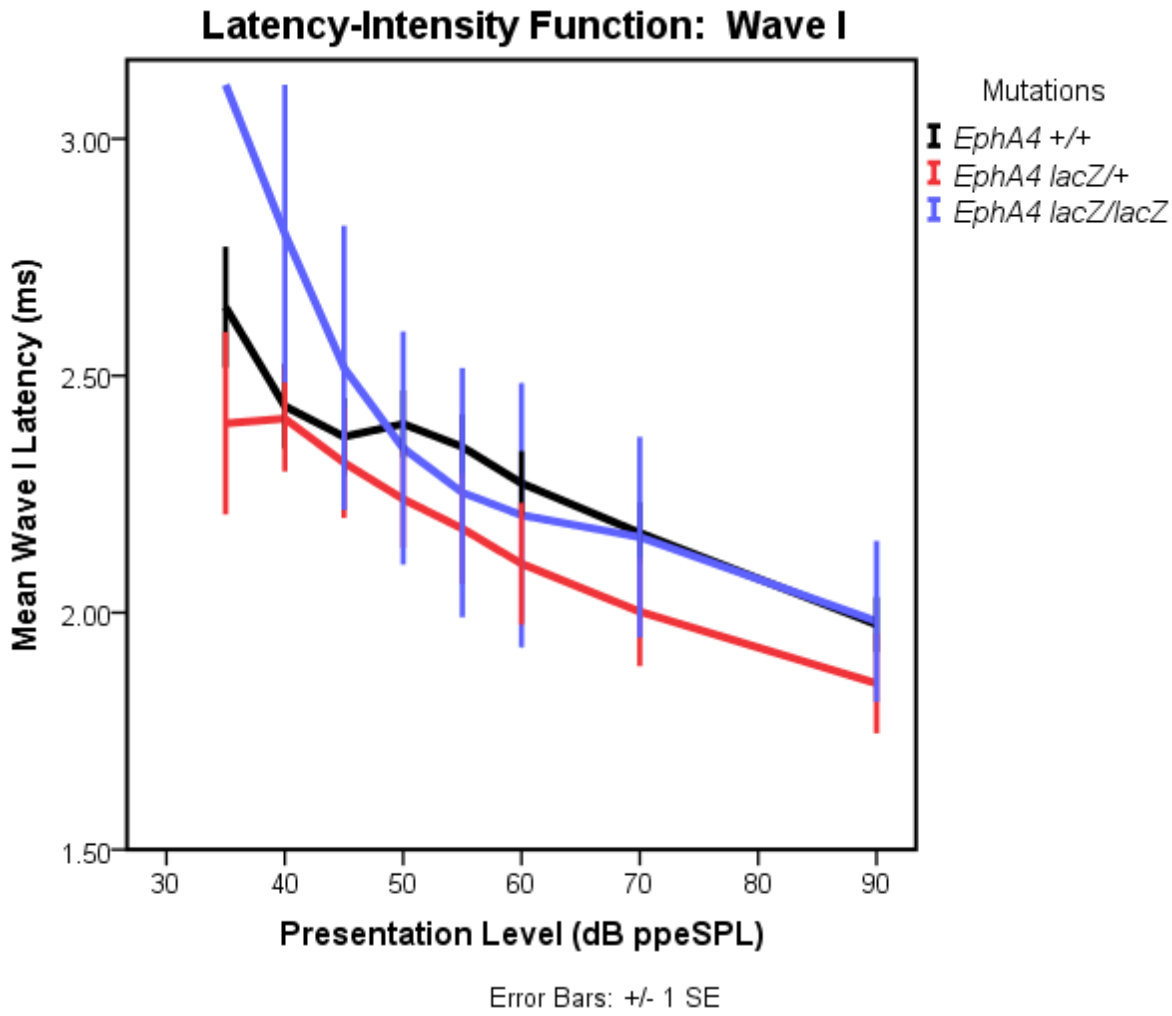


Figure 8. Wave I Latency-Intensity Function. Line graph showing wave 1 latency as a function of presentation level.

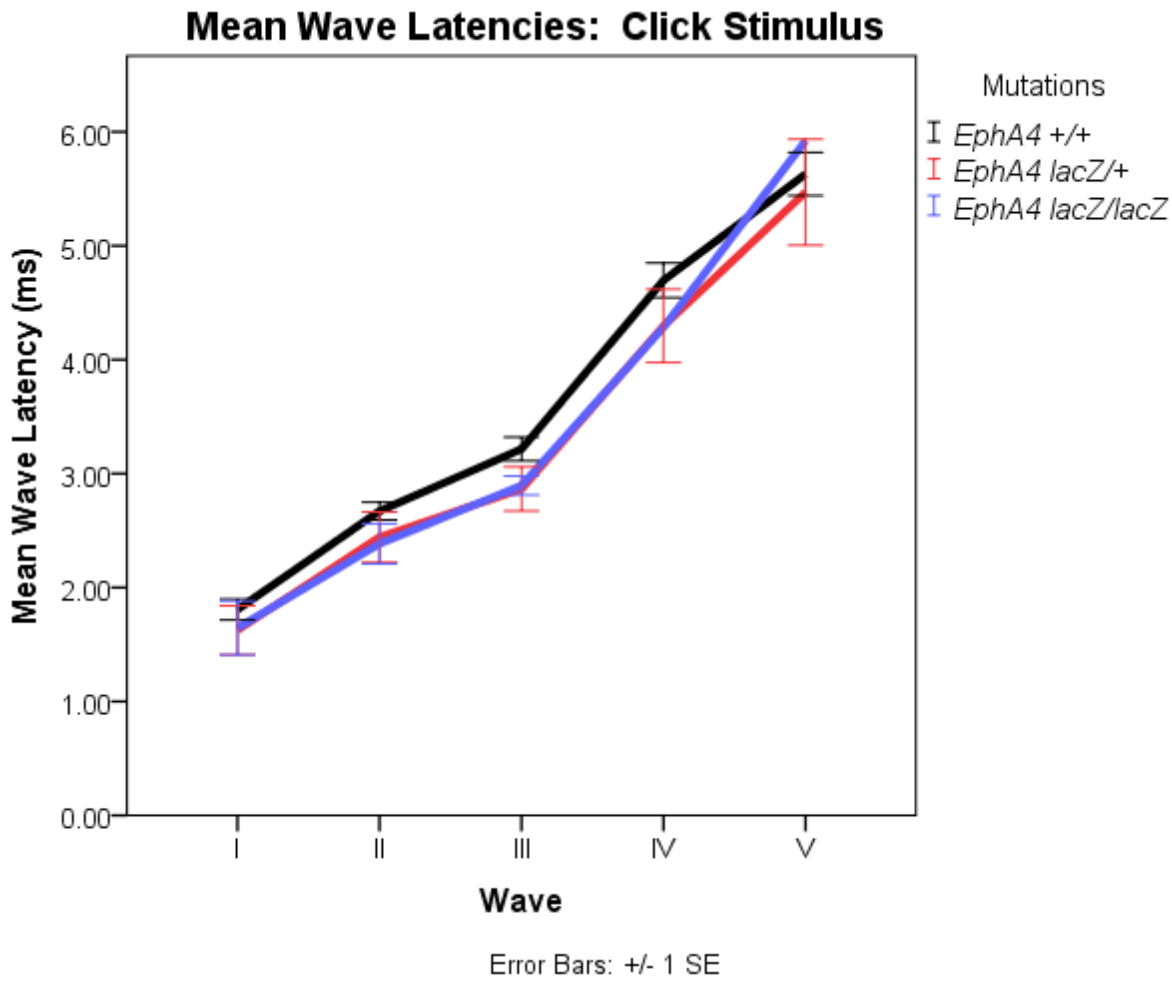


Figure 9. Line graph showing mean wave latency for waves I-V of each experimental group for click stimulus.

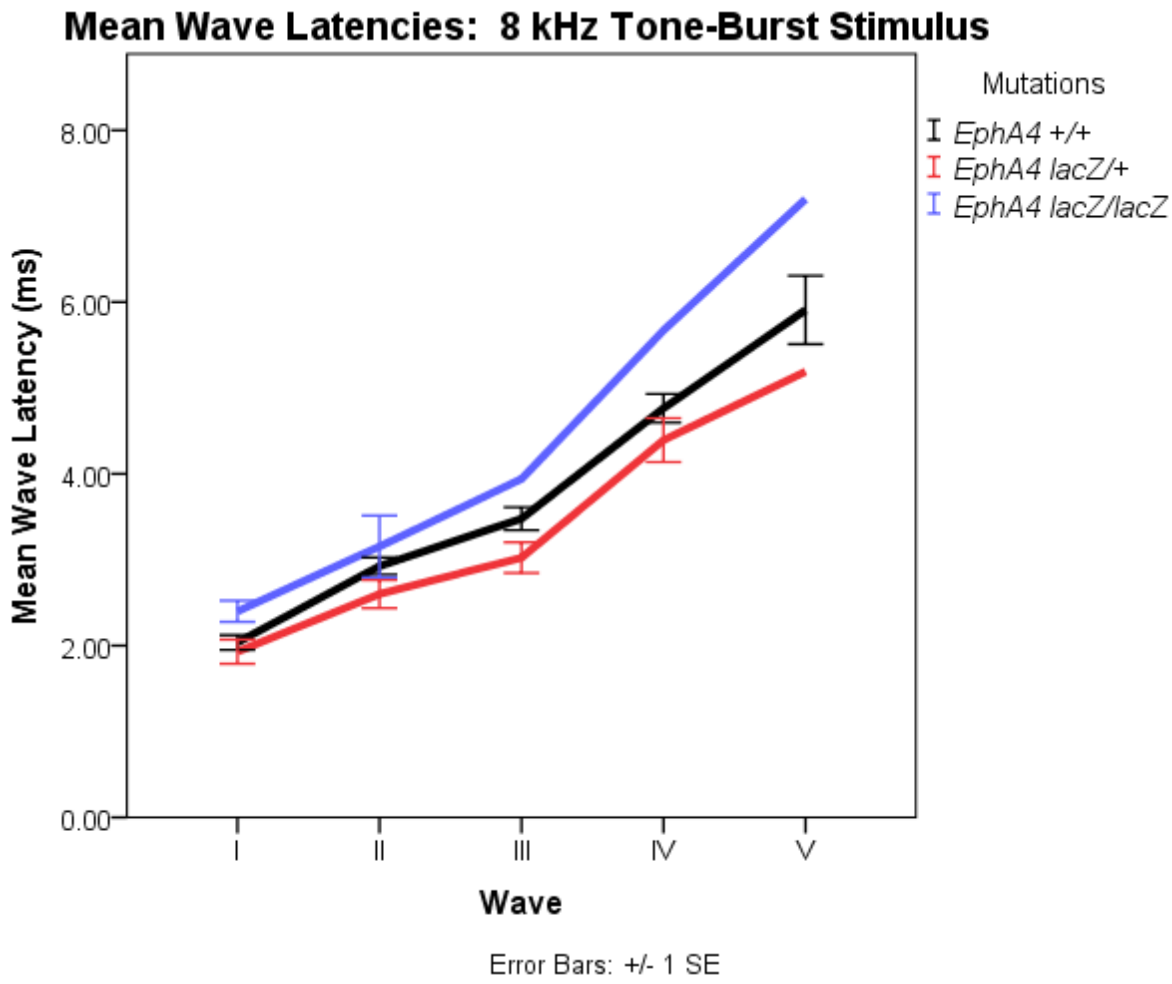


Figure 10. Line graph showing mean wave latency for waves I-V of each experimental group for 8 kHz tone-burst stimulus.

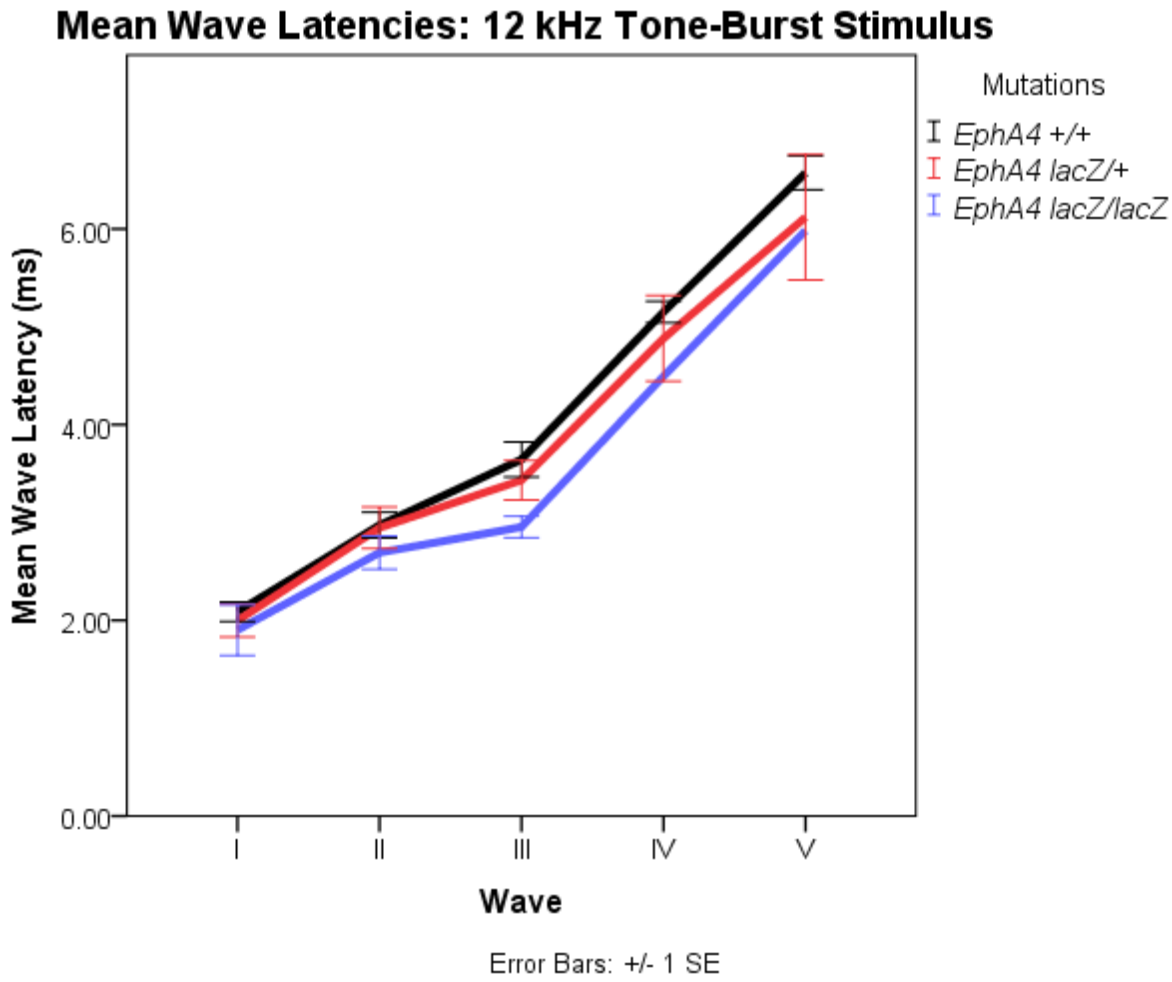


Figure 11. Line graph showing mean wave latency for waves I-V of each experimental group for 12 kHz tone-burst stimulus.

Average ABR Wave Latencies (ms)

	Click			8 kHz Tone-Burst			12 kHz Tone-Burst		
	+/+	+/*lacZ*	*lacZ/ lacZ*	+/+	+/*lacZ*	*lacZ/ lacZ*	+/+	+/*lacZ*	*lacZ/ lacZ*
I	1.81 (+- .32)	1.63 (+- .37)	1.65 (+- .34)	2.04 (+- .30)	1.93 (+- .25)	2.40 (+- .17)	2.09 (+- .35)	2.00 (+- .29)	1.90 (+- .37)
II	2.67 (+- .27)	2.44 (+- .38)	2.38 (+- .25)	2.93 (+- .33)	2.60 (+- .28)	3.15 (+- .51)	2.97 (+- .46)	2.95 (+- .37)	2.69 (+- .24)
III	3.46 (+- .30)	3.29 (+- .29)	3.40 (+- .004)	3.79 (+- .37)	3.45 (+- .34)	4.36 (*)	4.06 (+- .41)	3.92 (+- .32)	3.60 (*)
IV	4.70 (+- .48)	4.30 (+- .56)	4.28 (*)	4.76 (+- .50)	4.40 (+- .36)	5.67 (*)	5.15 (+- .33)	4.88 (+- .62)	4.49 (*)
V	5.63 (+- .46)	5.47 (+- .66)	5.92 (*)	5.91 (+- .80)	5.19 (*)	7.20 (*)	6.57 (+- .49)	6.12 (+- .91)	5.98 (*)

Table 2. Average ABR wave latencies (ms) for EphA$^{+/+}$, EphA4$^{+/lacZ}$, and EphA4$^{lacZ/lacZ}$ mice for wave I, II, III, IV, and V.

SPSS Output

SPSS Output: ANOVA of ABR Thresholds

```
GET   FILE='L:\Gerringer\FinalArchives\zHThresh.sav'.
GLM dBPEL.1 dBPEL.2 dBPEL.3 by Mutations
  /WSFACTOR=stim 3 Polynomial  /MEASURE=PEL
  /METHOD=SSTYPE(3)  /POSTHOC=Mutations(LSD)
  /PLOT=PROFILE(Mutations*Stim)  /PRINT=ETASQ
  /CRITERIA=ALPHA(.05)  /WSDESIGN=stim   /design=Mutations.
```

Within-Subjects Factors

Measure: PEL

stim	Dependent Variable
1	dBPEL.1
2	dBPEL.2
3	dBPEL.3

Between-Subjects Factors

		N
Mutations	0	11
	1	3
	2	3

Multivariate Tests[a]

Effect		Value	F	Hypothesis df	Error df	Sig.	Partial Eta Squared
stim	Pillai's Trace	.464	5.632[b]	2.000	13.000	.017	.464
	Wilks' Lambda	.536	5.632[b]	2.000	13.000	.017	.464
	Hotelling's Trace	.866	5.632[b]	2.000	13.000	.017	.464
	Roy's Largest Root	.866	5.632[b]	2.000	13.000	.017	.464
stim * Mutations	Pillai's Trace	.213	.833	4.000	28.000	.516	.106
	Wilks' Lambda	.795	.788[b]	4.000	26.000	.543	.108
	Hotelling's Trace	.247	.742	4.000	24.000	.573	.110
	Roy's Largest Root	.196	1.369[c]	2.000	14.000	.286	.164

a. Design: Intercept + Mutations Within Subjects Design: stim

b. Exact statistic

c. The statistic is an upper bound on F that yields a lower bound on the significance level.

Mauchly's Test of Sphericity[a]

Measure: PEL

Within Subjects	Mauchly's	Approx. Chi-	df	Sig.	Epsilon[b]

Effect	W	Square			Greenhouse-Geisser	Huynh-Feldt	Lower-bound
stim	.928	.965	2	.617	.933	1.000	.500

Tests the null hypothesis that the error covariance matrix of the orthonormalized transformed dependent variables is proportional to an identity matrix.

a. Design: Intercept + Mutations

Within Subjects Design: stim

b. May be used to adjust the degrees of freedom for the averaged tests of significance. Corrected tests are displayed in the Tests of Within-Subjects Effects table.

Tests of Within-Subjects Effects

Measure: PEL

Source		Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
stim	Sphericity Assumed	988.353	2	494.176	7.373	.003	.345
	Greenhouse-Geisser	988.353	1.866	529.534	7.373	.003	.345
	Huynh-Feldt	988.353	2.000	494.176	7.373	.003	.345
	Lower-bound	988.353	1.000	988.353	7.373	.017	.345
stim * Mutations	Sphericity Assumed	239.045	4	59.761	.892	.482	.113
	Greenhouse-Geisser	239.045	3.733	64.037	.892	.477	.113
	Huynh-Feldt	239.045	4.000	59.761	.892	.482	.113
	Lower-bound	239.045	2.000	119.522	.892	.432	.113
Error(stim)	Sphericity Assumed	1876.641	28	67.023			
	Greenhouse-Geisser	1876.641	26.130	71.818			
	Huynh-Feldt	1876.641	28.000	67.023			
	Lower-bound	1876.641	14.000	134.046			

Tests of Within-Subjects Contrasts

Measure: PEL

Source	stim	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
stim	Linear	898.500	1	898.500	11.956	.004	.461
	Quadratic	89.852	1	89.852	1.526	.237	.098
stim * Mutations	Linear	196.446	2	98.223	1.307	.302	.157
	Quadratic	42.599	2	21.299	.362	.703	.049
Error(stim)	Linear	1052.083	14	75.149			
	Quadratic	824.558	14	58.897			

Tests of Between-Subjects Effects

Measure: PEL

Transformed Variable: Average

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Intercept	108143.368	1	108143.368	219.739	.000	.940
Mutations	3819.288	2	1909.644	3.880	.046	.357
Error	6890.025	14	492.145			

Post Hoc Tests Mutations **Multiple Comparisons**

Measure: PEL

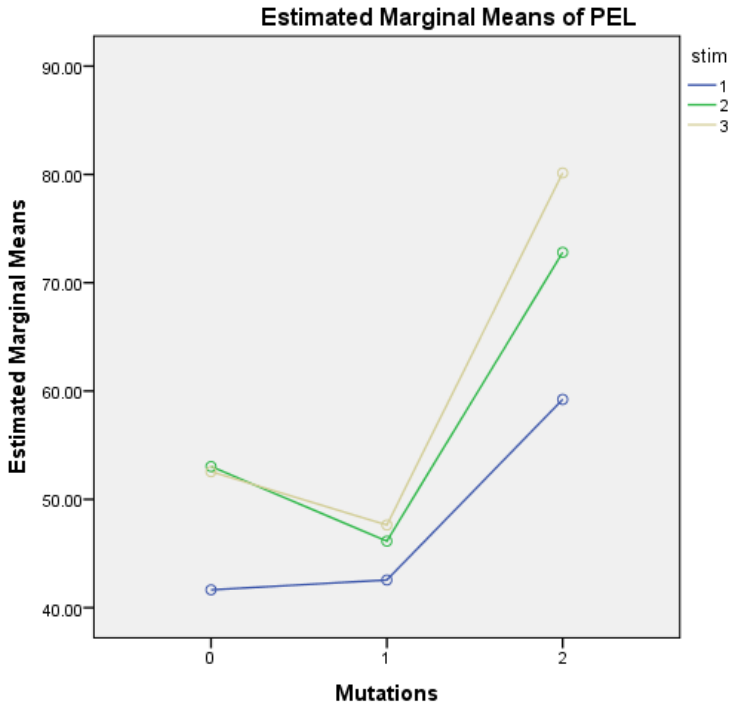
LSD

(I) Mutations	(J) Mutations	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
0	1	3.6364	8.34243	.670	-14.2564	21.5291
	2	-21.6414*	8.34243	.021	-39.5342	-3.7487
1	0	-3.6364	8.34243	.670	-21.5291	14.2564
	2	-25.2778*	10.45780	.030	-47.7075	-2.8480
2	0	21.6414*	8.34243	.021	3.7487	39.5342
	1	25.2778*	10.45780	.030	2.8480	47.7075

Based on observed means. The error term is Mean Square(Error) = 164.048.

*. The mean difference is significant at the .05 level.

Profile Plots



SPSS Output: ANCOVA of Presentation Level Effects on Wave I Latency

```
L:\Gerringer\FinalArchives\zHABR.sav
UNIANOVA P1ave BY STIM Mutations WITH dB
  /METHOD=SSTYPE(3)  /INTERCEPT=INCLUDE  /PLOT=PROFILE(Mutations*STIM)
/PRINT=ETASQ   /CRITERIA=ALPHA(.05)   /DESIGN=STIM Mutations dB
Mutations*STIM STIM*dB Mutations*dB Mutations*STIM*dB.
```

Between-Subjects Factors

		N
STIM	C	99
	E	96
	T	86
Mutations	0	193
	1	59
	2	29

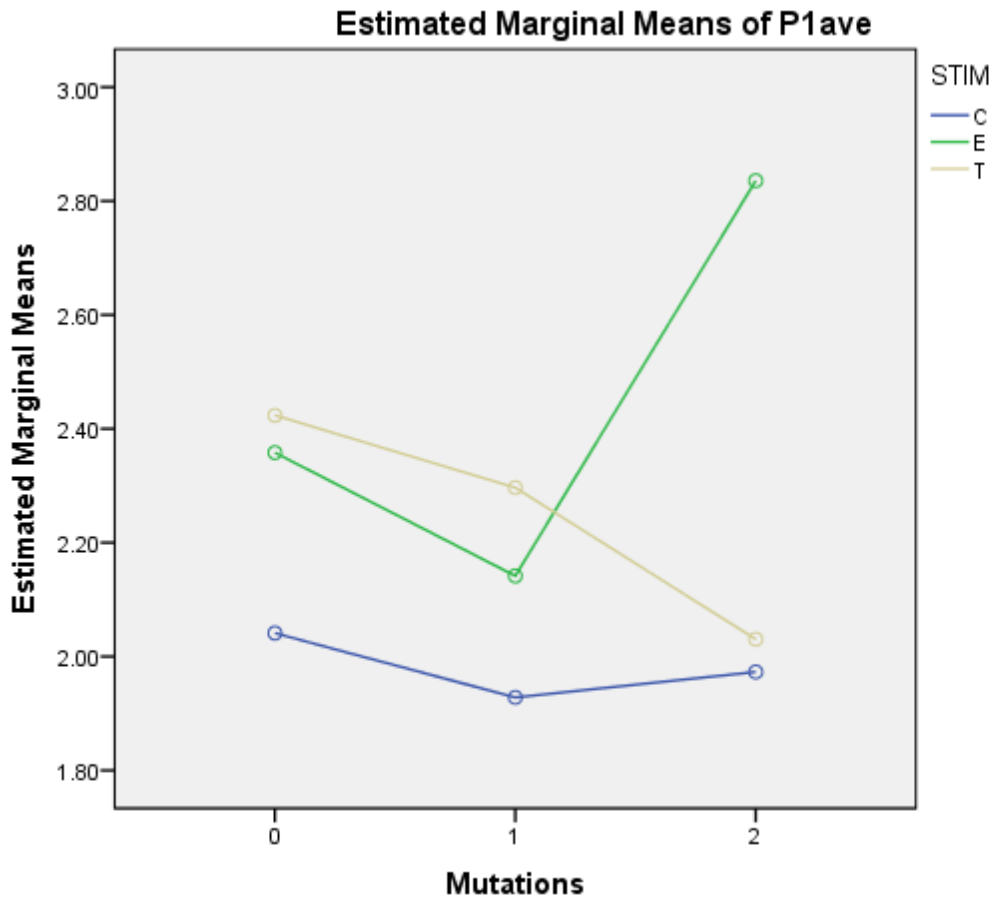
Tests of Between-Subjects Effects

Dependent Variable: P1ave

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	20.580[a]	17	1.211	13.440	.000	.465
Intercept	77.116	1	77.116	856.194	.000	.765
STIM	.307	2	.153	1.703	.184	.013
Mutations	.088	2	.044	.491	.613	.004
dB	4.542	1	4.542	50.427	.000	.161
STIM * Mutations	.689	4	.172	1.912	.109	.028
STIM * dB	.035	2	.018	.197	.822	.001
Mutations * dB	.005	2	.003	.030	.971	.000
STIM * Mutations * dB	.239	4	.060	.664	.618	.010
Error	23.688	263	.090			
Total	1453.157	281				
Corrected Total	44.268	280				

a. R Squared = .465 (Adjusted R Squared = .430)

Profile Plots



Covariates appearing in the model are evaluated at the following values: dB = 61.01

SPSS Output: ANCOVA of Absolute Wave Latency

```
[DataSet1] L:\Gerringer\FinalArchives\RestructuredMean5Peaks.sav

UNIANOVA latency BY STIM Mutations WITH Wave
  /METHOD=SSTYPE(3)
  /INTERCEPT=INCLUDE
  /PRINT=ETASQ
  /CRITERIA=ALPHA(.05)
  /DESIGN=STIM Mutations Wave Mutations*STIM Mutations*Wave STIM*Wave
Mutations*STIM*Wave.
```

Between-Subjects Factors

		N
STIM	C	74
	E	66
	T	73
Mutations	0	151
	1	39
	2	23

Tests of Between-Subjects Effects

Dependent Variable: latency

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	384.541[a]	17	22.620	107.083	.000	.903
Intercept	14.457	1	14.457	68.437	.000	.260
STIM	.796	2	.398	1.885	.155	.019
Mutations	.109	2	.055	.259	.772	.003
Wave	201.910	1	201.910	955.836	.000	.831
STIM * Mutations	.136	4	.034	.161	.958	.003
Mutations * Wave	.481	2	.240	1.137	.323	.012
STIM * Wave	.121	2	.061	.287	.751	.003
STIM * Mutations * Wave	1.176	4	.294	1.392	.238	.028
Error	41.192	195	.211			
Total	2997.830	213				
Corrected Total	425.733	212				

a. R Squared = .903 (Adjusted R Squared = .895)

SPSS Output: Repeated Measures ANOVA of Summating Potential Amplitude

```
[DataSet1] L:\Gerringer\FinalArchives\CMSP\zhSPCM.sav
GLM SPMag.1 SPMag.2 SPMag.3 BY Mutations
  /WSFACTOR=stimulus 3 Polynomial
  /MEASURE=SPmag   /METHOD=SSTYPE(3)   /POSTHOC=Mutations(LSD)
  /PLOT=PROFILE(Mutations*stimulus)  /PRINT=ETASQ
/CRITERIA=ALPHA(.05)
  /WSDESIGN=stimulus   /DESIGN=Mutations.
```

Warnings

Post hoc tests are not performed for Mutations because at least one group has fewer than two cases.

Within-Subjects Factors

Measure: SPmag

stimulus	Dependent Variable
1	SPMag.1
2	SPMag.2
3	SPMag.3

Between-Subjects Factors

		N
Mutations	0	8
	1	3
	2	1

Multivariate Tests[a]

Effect		Value	F	Hypothesis df	Error df	Sig.	Partial Eta Squared
stimulus	Pillai's Trace	.441	3.152[b]	2.000	8.000	.098	.441
	Wilks' Lambda	.559	3.152[b]	2.000	8.000	.098	.441
	Hotelling's Trace	.788	3.152[b]	2.000	8.000	.098	.441
	Roy's Largest Root	.788	3.152[b]	2.000	8.000	.098	.441
stimulus * Mutations	Pillai's Trace	.481	1.426	4.000	18.000	.266	.241
	Wilks' Lambda	.548	1.404[b]	4.000	16.000	.277	.260
	Hotelling's Trace	.772	1.351	4.000	14.000	.300	.278
	Roy's Largest Root	.695	3.127[c]	2.000	9.000	.093	.410

a. Design: Intercept + Mutations Within Subjects Design: stimulus

b. Exact statistic

c. The statistic is an upper bound on F that yields a lower bound on the significance level.

Mauchly's Test of Sphericity[a]

Measure: SPmag

Within Subjects Effect	Mauchly's W	Approx. Chi-Square	df	Sig.	Epsilon[b]		
					Greenhouse-Geisser	Huynh-Feldt	Lower-bound
stimulus	.655	3.379	2	.185	.744	1.000	.500

Tests the null hypothesis that the error covariance matrix of the orthonormalized transformed dependent variables is proportional to an identity matrix.

a. Design: Intercept + Mutations

Within Subjects Design: stimulus

b. May be used to adjust the degrees of freedom for the averaged tests of significance. Corrected tests are displayed in the Tests of Within-Subjects Effects table.

Tests of Within-Subjects Effects

Measure: SPmag

Source		Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
stimulus	Sphericity Assumed	441247.154	2	220623.577	5.587	.013	.383
	Greenhouse-Geisser	441247.154	1.487	296639.092	5.587	.024	.383
	Huynh-Feldt	441247.154	2.000	220623.577	5.587	.013	.383
	Lower-bound	441247.154	1.000	441247.154	5.587	.042	.383
stimulus * Mutations	Sphericity Assumed	331964.069	4	82991.017	2.102	.123	.318
	Greenhouse-Geisser	331964.069	2.975	111585.445	2.102	.148	.318
	Huynh-Feldt	331964.069	4.000	82991.017	2.102	.123	.318
	Lower-bound	331964.069	2.000	165982.035	2.102	.178	.318
Error(stimulus)	Sphericity Assumed	710748.177	18	39486.010			
	Greenhouse-Geisser	710748.177	13.387	53090.854			
	Huynh-Feldt	710748.177	18.000	39486.010			
	Lower-bound	710748.177	9.000	78972.020			

Tests of Within-Subjects Contrasts

Measure: SPmag

Source	stimulus	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
stimulus	Linear	204188.296	1	204188.296	5.762	.040	.390
	Quadratic	237058.858	1	237058.858	5.445	.044	.377
stimulus * Mutations	Linear	65017.979	2	32508.989	.917	.434	.169

	Quadratic	266946.091	2	133473.045	3.066	.097	.405
Error(stimulus)	Linear	318927.290	9	35436.366			
	Quadratic	391820.887	9	43535.654			

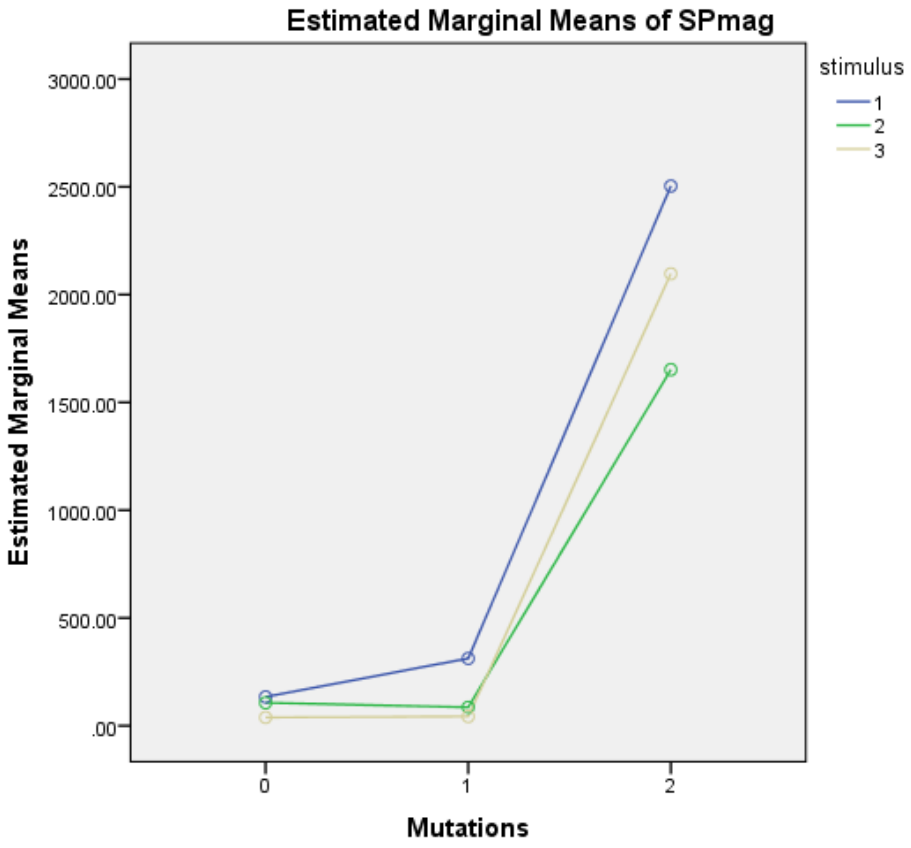
Tests of Between-Subjects Effects

Measure: SPmag

Transformed Variable: Average

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Intercept	11113634.537	1	11113634.537	1135.555	.000	.992
Mutations	10761408.201	2	5380704.100	549.783	.000	.992
Error	88082.698	9	9786.966			

Profile Plots



SPSS Output: MANOVA of SP/AP Ratio

```
[DataSet2] L:\Gerringer\FinalArchives\hAPSP.sav
GLM SPtoAPratio.1 SPtoAPratio.2 SPtoAPratio.3 BY Mutations
  /WSFACTOR=stim 3 Polynomial   /MEASURE=SPAPratio
  /METHOD=SSTYPE(3)   /PLOT=PROFILE(Mutations*stim)
  /PRINT=ETASQ   /CRITERIA=ALPHA(.05)   /WSDESIGN=stim
  /DESIGN=Mutations.
```

Within-Subjects Factors

Measure: SPAPratio

stim	Dependent Variable
1	SPtoAPratio.1
2	SPtoAPratio.2
3	SPtoAPratio.3

Between-Subjects Factors

		N
Mutations	0	8
	1	3
	2	1

Multivariate Tests[a]

Effect		Value	F	Hypothesis df	Error df	Sig.	Partial Eta Squared
stim	Pillai's Trace	.871	26.888[b]	2.000	8.000	.000	.871
	Wilks' Lambda	.129	26.888[b]	2.000	8.000	.000	.871
	Hotelling's Trace	6.722	26.888[b]	2.000	8.000	.000	.871
	Roy's Largest Root	6.722	26.888[b]	2.000	8.000	.000	.871
stim * Mutations	Pillai's Trace	.880	3.536	4.000	18.000	.027	.440
	Wilks' Lambda	.160	6.013[b]	4.000	16.000	.004	.601
	Hotelling's Trace	5.017	8.780	4.000	14.000	.001	.715
	Roy's Largest Root	4.967	22.353[c]	2.000	9.000	.000	.832

a. Design: Intercept + Mutations

Within Subjects Design: stim

b. Exact statistic

c. The statistic is an upper bound on F that yields a lower bound on the significance level.

Mauchly's Test of Sphericity[a]

Measure: SPAPratio

Within Subjects Effect	Mauchly's W	Approx. Chi-Square	df	Sig.	Epsilon[b]		
					Greenhouse-Geisser	Huynh-Feldt	Lower-bound
stim	.611	3.936	2	.140	.720	1.000	.500

Tests the null hypothesis that the error covariance matrix of the orthonormalized transformed dependent variables is proportional to an identity matrix.

a. Design: Intercept + Mutations

Within Subjects Design: stim

b. May be used to adjust the degrees of freedom for the averaged tests of significance. Corrected tests are displayed in the Tests of Within-Subjects Effects table.

Tests of Within-Subjects Effects

Measure: SPAPratio

Source		Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
stim	Sphericity Assumed	.247	2	.124	21.185	.000	.702
	Greenhouse-Geisser	.247	1.440	.172	21.185	.000	.702
	Huynh-Feldt	.247	2.000	.124	21.185	.000	.702
	Lower-bound	.247	1.000	.247	21.185	.001	.702
stim * Mutations	Sphericity Assumed	.245	4	.061	10.505	.000	.700
	Greenhouse-Geisser	.245	2.881	.085	10.505	.001	.700
	Huynh-Feldt	.245	4.000	.061	10.505	.000	.700
	Lower-bound	.245	2.000	.123	10.505	.004	.700
Error(stim)	Sphericity Assumed	.105	18	.006			
	Greenhouse-Geisser	.105	12.963	.008			
	Huynh-Feldt	.105	18.000	.006			
	Lower-bound	.105	9.000	.012			

Tests of Within-Subjects Contrasts

Measure: SPAPratio

Source	stim	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
stim	Linear	.185	1	.185	59.512	.000	.869
	Quadratic	.063	1	.063	7.330	.024	.449
stim * Mutations	Linear	.138	2	.069	22.258	.000	.832
	Quadratic	.107	2	.054	6.256	.020	.582

Error(stim)	Linear	.028	9	.003			
	Quadratic	.077	9	.009			

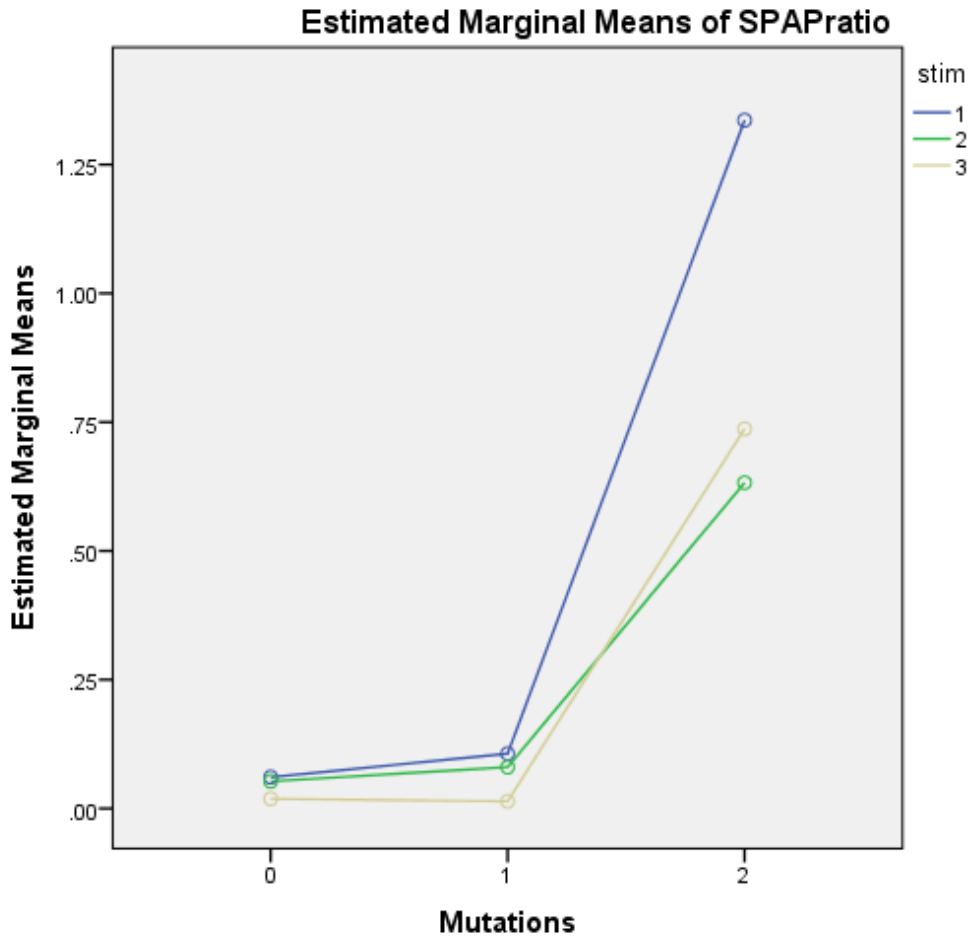
Tests of Between-Subjects Effects

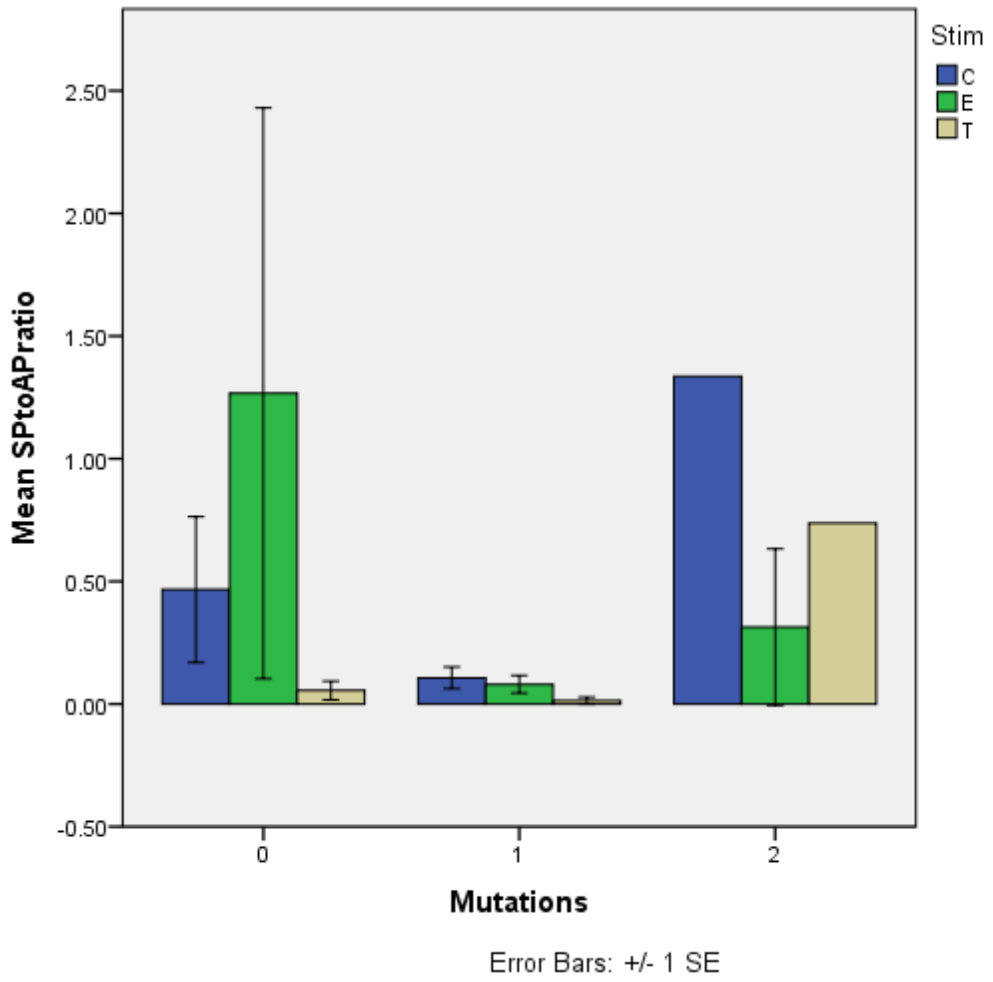
Measure: SPAPratio

Transformed Variable: Average

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Intercept	2.110	1	2.110	614.302	.000	.986
Mutations	1.998	2	.999	290.816	.000	.985
Error	.031	9	.003			

Profile Plots




Stim
C
E
T
Mean SPtoAPratio
2.50
2.00
1.50
1.00
0.50
0.00
-0.50
0
1
2
Mutations
Error Bars: +/- 1 SE

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