Spring 2013

Ultrasonic Vocalizations in Eph/ephrin Mutant Mice

Bryna Rickenbach-Cline
James Madison University

Ultrasonic Vocalizations in Eph/ephrin Mutant Mice

Bryna Lee Rickenbach-Cline

A dissertation submitted to the Graduate Faculty of

JAMES MADISON UNIVERSITY

In

Partial fulfillment of the Requirements

for the degree of

Doctor of Audiology

Communication Sciences and Disorders

May 2013

Dedication

To my husband, Bill, who is a constant source of encouragement. Thank you for believing in me and keeping me motivated through many late nights and long days of coursework and clinical placements. Without the endless supply of tea that you provided I would have never made it to class on time let alone through the last four years!

To my parents, Gary and Jamie, for instilling in me a work ethic and courage that has taken me beyond my wildest dreams. Thank you both for providing for me the means to an education and for offering your love and support from the very beginning.

To my grandparents, Edwin and Alice Rickenbach and John and Geraldine Heckman, who helped provide the financial means to cover my expenses during graduate school. Whether here to see my graduation or watching from above; thank you for the love and support you have shown to me in all of my adventures.

To my sister and best friend, Kailyn White, who was always available to listen and provide a fresh perspective on what seemed like an endless number of nearly impossible hurdles to complete. Thank you for always believing in your big sister.

Acknowledgements

With my most sincere appreciation to my committee chair Dr. Lincoln Gray; the most well-rounded individual with whom I have ever had the pleasure of working. Dr. Gray's enthusiasm for teaching, learning, and living is apparent in each encounter and conversation one may have with him. Without his genius as a researcher and statistician, knack for computer programming, and never-ending encouragement this dissertation would not have been possible.

Thank you to my committee members, Dr. Mark Gabriele and Dr. Brenda Ryals, whose commitment to research and scholarship contributes so much to their respective fields.

With appreciation to Dr. Christy Ludlow and Dr. Rory DePaolis whose contributions to my literature review greatly helped improve my understanding of anatomical structures and the development of infant vocalizations and how these topics relate to my research.

My most sincere gratitude to Lauren O'Baugh, Jessica Green, and Logan Douglas; students in the Communication Sciences and Disorders department who so graciously donated their time to corral the tiny mouse subjects and assisted in the collection of data. Continued thanks to Sara Kavianpour, Matt Wallace, and many other students from the Biology department always available to genotype mouse litters, clean cages, and feed our ever-growing mouse empire.

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Abstract

Purpose: Murine mammals, more commonly known as mice, emit ultrasonic vocalizations or "yelps" to communicate. It has been found that adult mice use these vocalizations to communicate with each other, and infant mice utilize ultrasonic vocalizations to communicate with their mother. It is known that a relationship exists between hearing ability and vocalization (Bass-Ringdahl, 2010). This study aims to record yelps/ vocalizations of four strains of mouse pups to determine how the development of hearing relates to the vocalizations that are emitted. It is thought that mice with compromised genes that encode for signaling proteins that are essential to the development of auditory connections may emit abnormal vocalizations. Thus, it is possible that the earliest relevant behavior that predicts normal auditory development in mice is the emission of vocalizations of mouse pups.

Method: A total of one hundred fifty seven tests of pups between 7 and 26 days age were tested alone in a dark, sound-attenuating booth. The duration of the test time depended on the age of the pup. A B&K model 4939 ¼-inch microphone connected to an Agilent 35670 Spectrum Analyzer recorded all sounds between 10 and 100 kHz above a threshold (40 dB SPL – slightly above background). The yelps emitted were organized into four experimental groups, wild-type (WT), ephrin-B3^{null}, ephrin-B3^{lacZ}, and ephrin-B2^{lacZ}, based on the subject's genetic strain. A statistical analysis was performed to determine the yelp rate and pitch range of each group of mice.

Results: This study found that ephrin-B3^{null} (a traditional knockout) emit significantly more vocalizations than other groups. The ephrin-B3^{lacZ} group that still

expresses the protein but has compromised signaling had the fewest total vocalizations, but also the highest percentage of overall sounds in the range of the ultrasonic vocalizations.

Conclusions: The Eph/ephrin genotype, known to affect hearing, also affects neonatal vocalizations.

Chapter 1: Manuscript

Introduction

This study compares the vocalizations of wild-type (WT) mouse pups and three groups of transgenic mice. The Eph/ephrin family of signaling proteins is involved in the development of organized auditory afferents and the development of various parts of the auditory system (Miko et al., 2008). Three different mutations were studied: a traditional ephrin-B3 knockout (ephrin-B3^{null}) that completely lacks the signaling protein, and two *lacZ* mutants (ephrin-B3^{lacZ}) (ephrin-B2^{lacZ}) that are incapable of reverse signaling due to inactivation of a necessary cytoplasmic kinase domain. The vocalizations emitted by the mutant pups were compared to those of wild-type mice. Literature suggests a relationship between hearing ability and vocalization emission (Easterbrooks, & Baker, 2002), so mouse vocalizations were compared to see if these mutations, known to affect hearing (Miko et al., 2008) also affect the vocalization rate and pitch of the pups tested. All mice were housed in the CSD2 laboratory at James Madison University.

Research performed by Portfors (2007) suggests that ultrasonic vocalizations in wild-type mice are much different than vocalizations in genetically altered mice. Portfors clearly explains the importance of using wild-type mice as a control because the differences found will help researchers understand how various environmental conditions affect the diversity, complexity, and function of the ultrasonic vocalizations of the mouse. (Portfors, 2007)

Based on findings of prior research, in our comparison between the vocalization rate of wild-type Eph/ephrin mutants we expect to see a difference in vocalization rate between strains of mice. Keeping in mind the relationship that exists between

vocalization and hearing ability, it is possible that mutant mice that either lack or express compromised forms of essential signaling proteins for the development of the auditory system, may exhibit abnormal vocalizations as a result of abnormal hearing ability. In summary, the purposes of this research were to establish the following:

1) The differences in the vocalization rate of wild-type mice pups as compared to mice pups with Eph/ephrin mutations.
2) The effect of various genetic mutations on vocalization rate and pitch.
3) An attempt to separate vocalizations from other sounds, here called ‘yelps’ and ‘scuffles’.

Methods

Participants

One hundred fifty seven tests were completed of four different genotypes between 7 days and 26 days of age, all housed in the CSD2 Laboratory at James Madison University. The control strain consisted of the C57BL/6J strain of mouse originating from Jackson Labs. There is nothing unusual or altered in the Eph or ephrin genes of this control strain, making it a good choice for this study of how the established auditory circuits affect vocalization behaviors. It should be noted, however, that the C57BL/6J strain does not serve as an ideal control group for many behavioral studies as it is well established this strain exhibits early onset hearing loss (as early as postnatal day 90). Since all tests were performed of mice prior to this period, this was not a concern in this study.

The second strain used in this study was a traditional knockout for the ephrin-B3 protein, ephrin-B3^{null}. These mice exhibit a complete deletion of the ephrin-B3 gene and are thus termed "true knockouts." In a cross of heterozygous parents, one quarter of the progeny will be homozygous and completely lack the ephrin-B3 protein. Aside from exhibiting defects in developing auditory circuits, these pups are known to exhibit corticospinal motor dysfunction and exhibit a characteristic hopping hindlimb gait.

Another ephrin-B3 strain was utilized in this study, the ephrin-B3$^{lac\ Z}$ mouse. While similar to the ephrin-B3^{null} mouse, the ephrin-B3$^{lac\ Z}$ mouse still contains the ephrin-B3 gene. However, in *lacZ* mutants manipulations to the gene render the expressed protein incapable of its normal signaling function.

The final experimental group involved a similar *lacZ* mutant for the ephrin-B2 protein. Interestingly, only the heterozygous progeny of this strain were tested as the homozygotes of this strain are perinatally lethal.

The reason that these groups of mutant mice were chosen is the fact that Eph/ephrin genes are known to affect the auditory system (Gabriele et al., 2011; Wallace et al., 2013). Eph/ephrins are considered the largest family of signaling molecules that fall under the category of receptor tyrosine kinases. Eph/ephrin binding triggers many 2nd messenger systems allowing communication from outside to inside the cell. Neither Ephs nor ephrins are traditional receptors or ligands in that they are both membrane bound and are capable of signaling in both forward and reverse directions. Ephs are considered classic receptor molecules, while ephrins are the corresponding ligands that bind Ephs. Ephs must bind corresponding ephrins, or vice versa, and these interactions have been shown to result in attractive or repulsive responses depending on the system studied.

Another reason these ephrin mutants were chosen for this study is their involvement in the establishment of topographic patterns in the developing brain. Axonal inputs and Eph/ephrin interactions with their target cells assist in establishing topographical connections in the brain and, more specifically, the auditory system. Our colleagues' research (Gabriele, 2011) looks at how auditory connections are affected in Eph/ephrin mutants, and our research looks what effect this might have on their vocalization behaviors. Within the inferior colliculus, or auditory midbrain, Eph/ephrins are important in defining two types of maps: (1) continuous maps, where specific topographic maps such as the tonotopic organization within the auditory system are

created, as well as (2) discrete maps, where information from multiple converging sources target spatially discrete areas of the target. During development, axons invade the inferior colliculus and form both continuous and discrete connections prior to the onset of hearing (Gabriele et al., 2000, 2011; Wallace et al., 2013). Many of the instructive cues necessary for this early establishment of topographic projection patterns in the absence of experience come from Eph/ephrin protein signaling as well as correlated events of spontaneous activity in the immature cochlea.

The spontaneous activity mentioned above is generated by Kolliker's organ and through its release of ATP that synchronizes the depolarization of inner hair cells. These orchestrated events are conveyed up the ascending system and are required for the accurate establishment of the tonotopy and pattern formation in the developing system.

Though it has been found that there is an effect of the Eph/ephrin proteins in the development of the auditory system, there is a need to determine significance of their role in defining functional auditory circuits. The use of the described mutant mice will allow further research into the effect of these mutations on the hearing ability and vocalization behaviors. Human correlates of such findings can then be used to better understand the development of the human auditory system and the role it plays in vocalization production which is so important in speech and language development.

Figure 1: Mouse pup subject

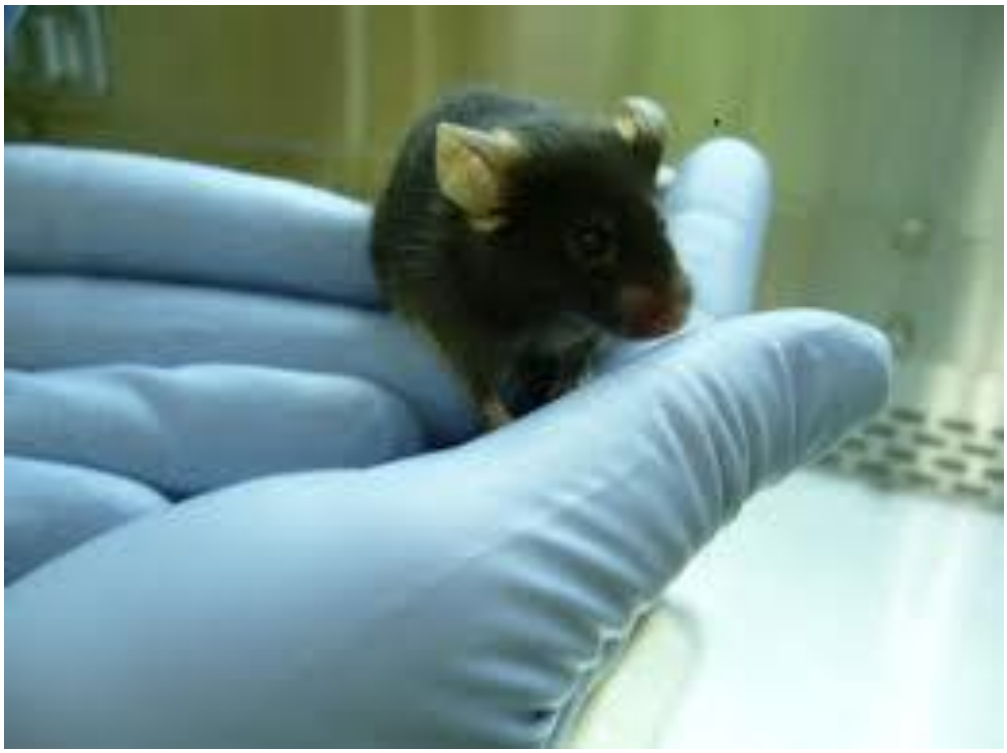


Figure 2: Litter of newborn mice

Procedure

All subjects were tested alone in a clean cage in a dark, sound-attenuating booth. The cage utilized during the testing measured 17 cm wide x 28 cm long x 13 cm high.

Figure 3: Test Chamber

The test chamber was cleaned prior to the testing of each subject in an effort to remove the presence of any scent from another mouse that may be unfamiliar to the subject. Each plastic cage was sanitized in a dishwasher prior to use. This precaution ensured that the vocalizations recorded from the pup were not influenced by the olfactory system. While an unknown scent may certainly evoke increased vocalization rate in the mouse pups tested, the study does not aim to measure the vocalization rate in response to a novel olfactory input. Therefore, every precaution was taken to reduce the chance that unrelated outside factors affected the responses measured during recording.

One factor that was varied during data collection was the duration of test time. The length of the recording varied depending on the age of the subject. For instance, pups that were 7 days old were tested for only 20 minutes while older pups such as those 17 days old were tested for one hour. Test duration was varied as a function of the age of the pup so as not to cause physical pain or harm to the subject. Because very young pups are highly dependent on their mother for protection and warmth due to the limited amount of fur on their bodies, young pups were tested for a shorter amount of time to prevent hypothermia. To further reduce the chance of hypothermia, a heating pad was placed under the plastic test chamber to keep the bodies of the pups at a safe temperature.

Due to the fact that the vocalizations of the mouse pups are inaudible to humans without the use of specialized equipment (Portfors, 2007), A B&K model 4939 ¼ inch microphone connected to an Agilent 35670 Spectrum Analyzer recorded all sounds between 10 and 100 kHz above a threshold (40 dB SPL – slightly above background).

A Matlab program constantly queried the spectrum analyzer for the x and y coordinates (Hz and dB) of the spectral peak. The speed of this recording is not known

but probably occurs many times a second. It appears that peaks we observe are recorded. For each peak over 40 dB the program recorded the dB, kHz and time in seconds from the start of the test. For each mouse tested there was a file consisting of one row for each occurrence of a peak in the recorded spectrum > 40 dB. There were a total of over 45,000 such occurrences in a total of 181 tests averaging 59 minutes per test. An average of 250 triggers was recorded from each test, in a range from 0 to 1644.

Statistical analyses were performed utilizing the Statistical Package for the Social Sciences (SPSS) Versions 18-20. Since mice were tested for different lengths of time, yelps and scuffles per hour are the primary dependent variables in the following analyses.

Results

Only sounds between 40 and 70 kHz were considered to be ultrasonic vocalizations or 'yelps', while all other sounds were considered to be 'scuffles,' or ambient noise created by the movement of the subject. Twenty-eight pups were ineligible to be included as part of the analyses due to the fact that they were considered to be extreme outliers because no vocalizations or scuffles were recorded during the test session.

In a One-way ANOVA of yelps/hr across the 4 groups there was a significant effect of group as seen in Fig 4 ($F_{3,154}$=9.5, p<.001 with LSD post-hoc tests showing ephrin-EB3^{null} different than other groups). In a separate 2-way ANOVA using age in quartiles and group as independent variables, the age-by-group interaction approached significance (p=.07), but the main effect of age was not significant (p=.67). The number of 'yelps' was correlated with the number of 'scuffles' (r^2=53%, p<.001) showing a likely effect of genotype on overall activity. Percent of total sounds that are in the range of ultrasonic vocalization is unrelated to total activity (yelps+scuffles) (r^2=1%, p=.13). The group (EB2^{lacZ}) that had the fewest number of total vocalization had the highest percent of sounds in the range of ultrasonic vocalizations as seen in Figure 5.

Analyses were also performed on the mice as divided by age quartile. Age quartiles were determined by separating the test subjects into four categories as defined by their age that contained equal numbers of tests. Age quartile one included the very youngest through subjects 10 days of age. Quartile two included mice aged 11 to 16 days old, and quartile three included mice from 17 to 20 days of age. Age quartile four

included mice from 21 days of age to the very oldest mouse tested. Figure 6, as shown below, is an analysis of all wild type mice and all mutant mice divided by age quartile. Figure 6 shows that while the vocalizations of the wild type mice peaked during age quartile three (~P19), the mutant mice vocalizations peaked during age quartile two (~P14). It can also be observed that the mutant mice seemingly vocalized more overall than the wild type mice, though since all mutant mice were categorized together it is unclear if this is due to the population as a whole or a sub-category of mutant mice that has skewed the data to show this effect.

Figure 4: Mean Number of Ultrasonic Vocalizations in Four Groups of Mouse Pups

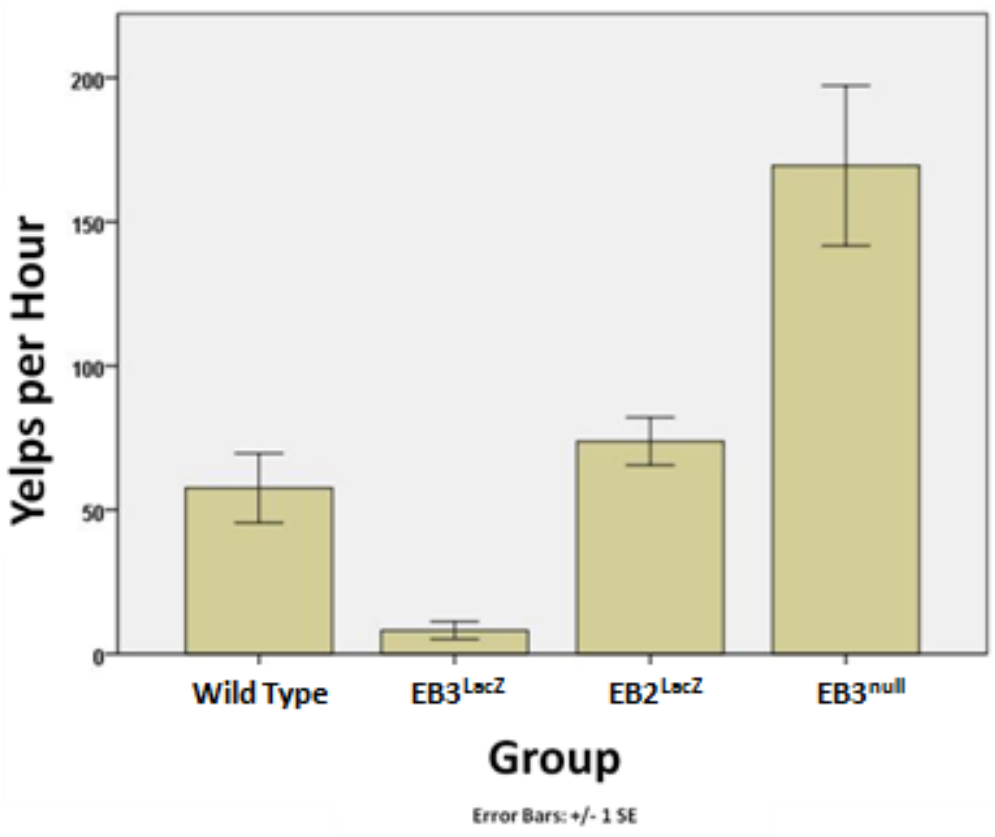


Figure 5: Percent of total triggers in frequency range of ultrasonic vocalizations

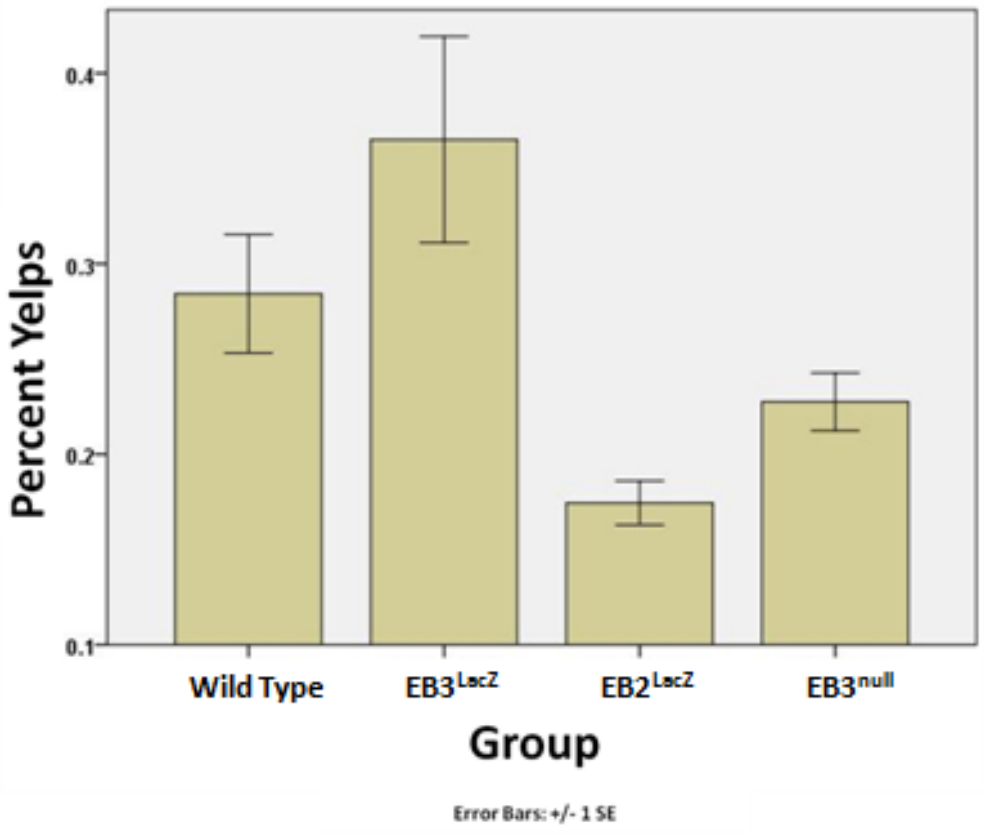
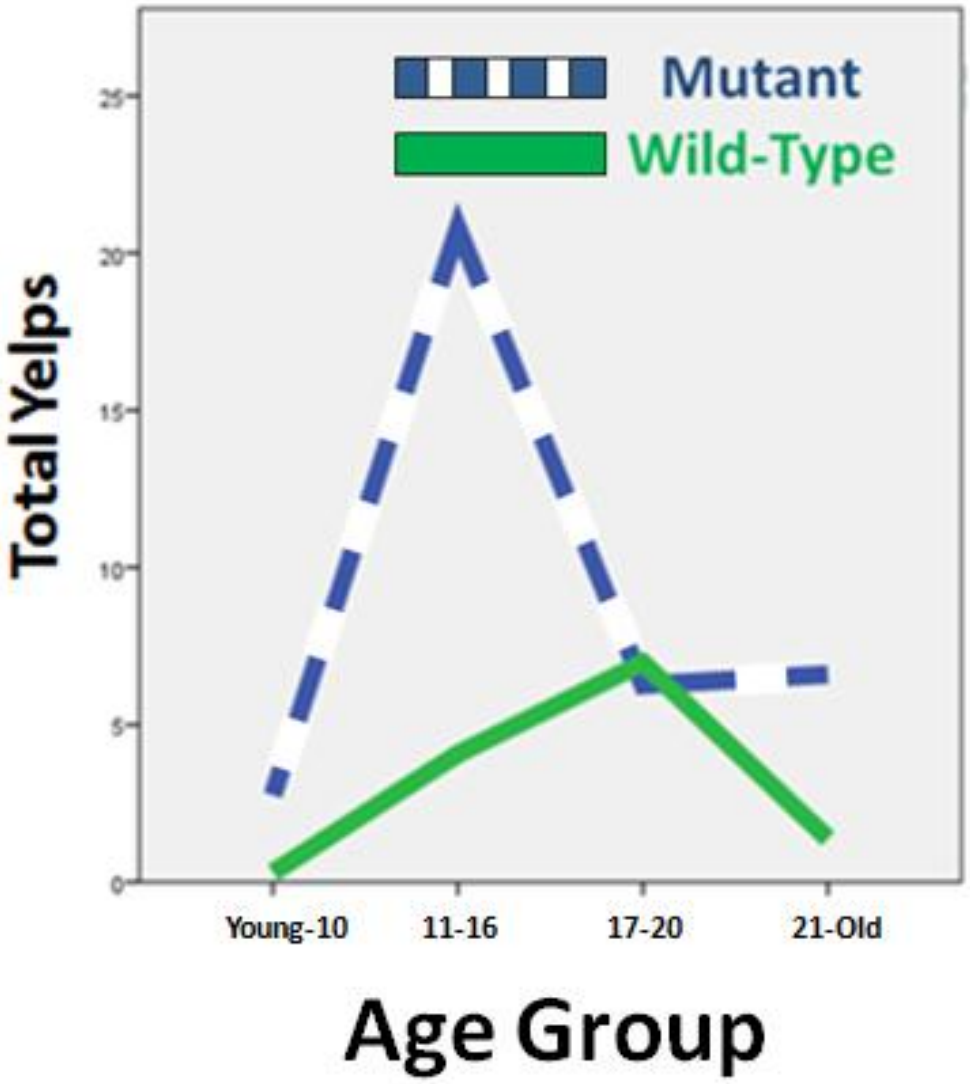


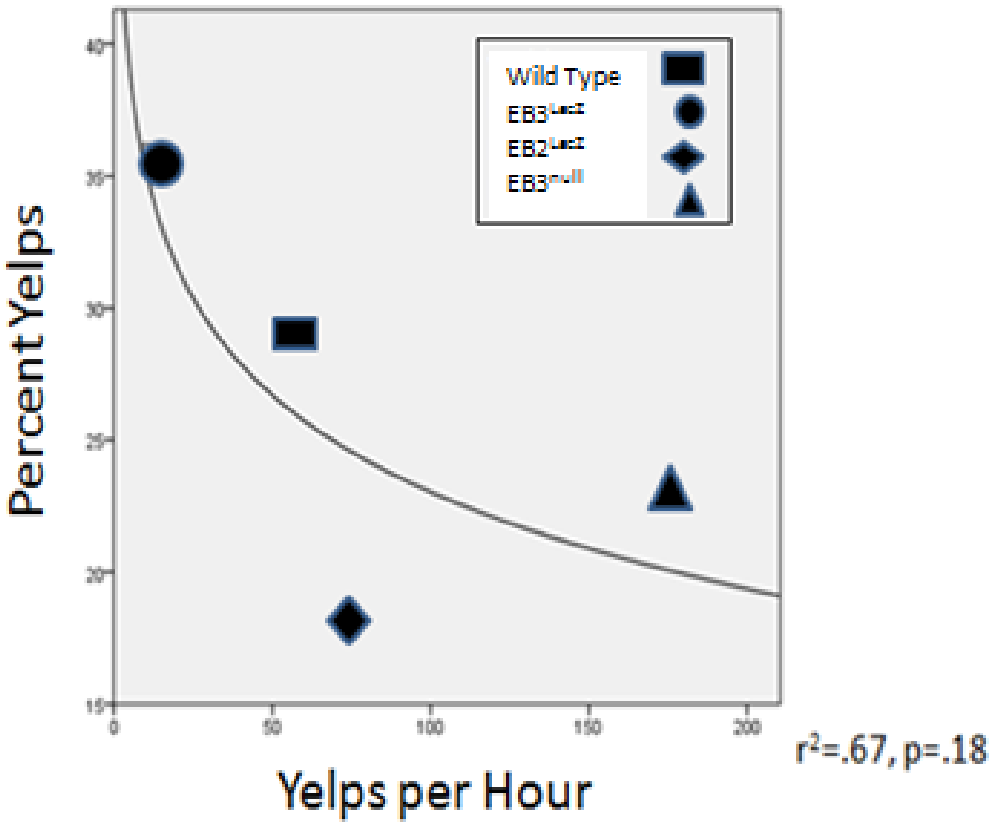
Figure 6: Vocalizations of wild type mice as compared to mutant mice as an effect of age



Discussion

It can be observed in Figures 4 and 5, above, that there is a significant difference between the groups in both yelps per hour and percent yelps. This shows that Eph/ephrin mutations have a significant effect on sounds that can be recorded from isolated pups, be those sounds vocalizations or scuffles. The two main outcome variables in this study (yelps per hour and percent yelps) are not significantly related ($p=.18$). Figure 7 plots the means of the data in Figures 4 and 5 for the 4 groups. The relationship approaches negativity, explaining two-thirds of the variance or a very large effect size. That is the more triggers, or sounds recorded, the fewer percent are actually yelps.

Figure 7: A graph comparing the means of the data in Figures 4 and 5 for the 4 groups of mice. This graph suggests that the more triggers that were recorded, the fewer percent of the triggers are actually vocalizations.



Despite the lack of relationship found, it was thought that the vocalizations of the various groups may be related to the hearing ability of the mice. A 2012 study by Shearer researched the hearing ability of various mouse strains. Therefore, a comparison was made between the results of Shearer's research (Shearer, 2012) on ABR measures of mice of various strains and the results of the present research based on murine vocalization production. Due to the fact that Shearer utilized twelve methods (described below) to determine hearing ability of the mice studied, some consideration as to the measure of hearing ability of the mice to be used for comparison purposes for this study was necessary. Instead of utilizing just one of the methods representing hearing ability, a composite score of the twelve methods utilized by Shearer was constructed. Figures 8 and 9 plot the number of yelps per hour and the percent yelps versus the composite score created from the results of the previous ABR study. Only 3 groups (wild type, and two ephrin-B3) were available for both studies. The composite score, termed zABR, was formed by first normalizing the data in Shearer's tables (thresholds, Wave I and V latencies, and Wave I amplitudes for clicks, 8kHz and 12 kHz tone pips). The z-scores for amplitudes were multiplied by -1, because we expect higher amplitudes to be associated with lower thresholds and faster latencies. A composite score constructed in this manner from these 12 Scores explained 80% of the variance with each of Shearer's measures contributing at least 89% to the composite. Figures 8 and 9 show that the composite score of Shearer's data compared to the two outcome variables of this study are convincingly unrelated ($p>0.01$)

Figure 8: Number of yelps per hour as compared to the composite score of hearing acuity found in Shearer's ABR study. It should be noted that a low zABR is indicative of good hearing (low thresholds, short latencies and high amplitudes indicate better hearing).

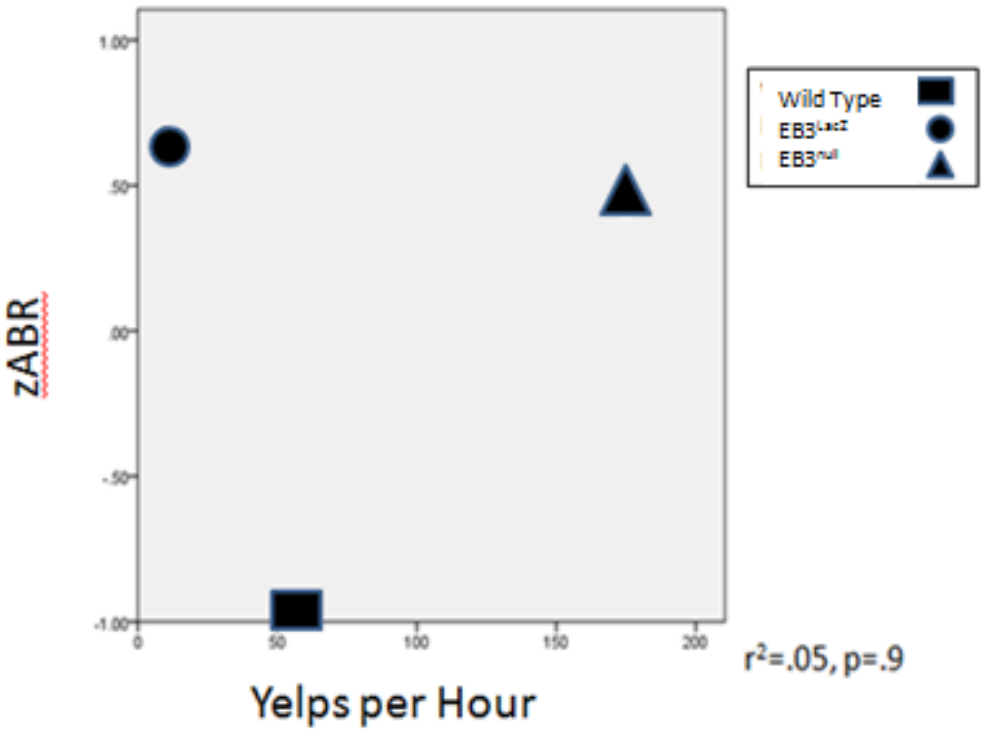
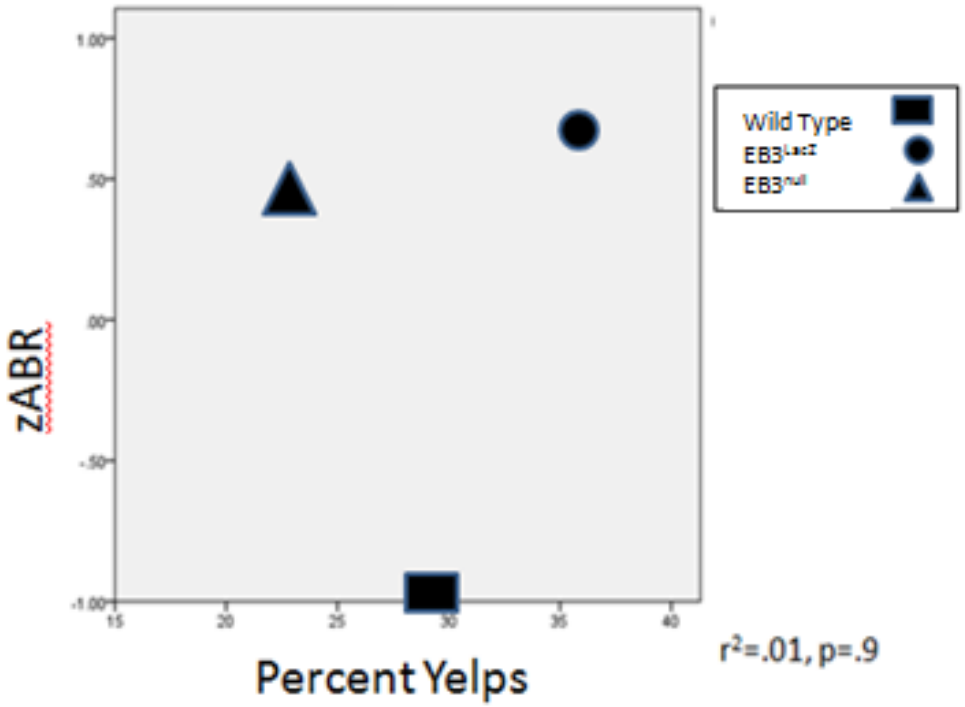


Figure 9: Percentage of vocalizations recorded as compared to the composite score of hearing acuity found in Shearer's ABR study. It should be noted that a low zABR is indicative of good hearing and that the lower the zABR the better the hearing of the subject will be (low thresholds and latencies indicate better hearing and amplitudes).



Due to the fact that no relationship was found between the two main outcome variables of this study and the hearing ability of the strains of mice, it is still unclear as to the reason for the differences in the behavioral measures shown in Figures 4 and 5. One possible conclusion of this study is that the effect can perhaps be attributed to motor activity. Though measures were taken to collect data logically and carefully, it was difficult to record the vocalizations of the mice without recording the "scuffles," or noise created due to the movements of the mice. The scuffles were easily recorded by the ultrasonic microphone utilized to record data. In this initial study, only the most conveniently automated method (criteria by frequency and intensity) was used to determine what was considered a vocalization or not. In reviewing test methods utilized by other researchers studying similar subjects, it was discovered that some investigators also testing mice in a polycarbonate cage coated the bottom and sides of the test chamber with a thin layer of paraffin wax to reduce the noise created by the subjects' claws against the cage. The wax is then melted, discarded, and the cage disinfected following each data collection session for the safety of the test subject when returned back to its litter (Warburton, 1987). It is our recommendation to anyone further exploring this area of research to utilize a similar method in an attempt to differentiate whether a recorded "hit" should be considered a vocalization or arbitrary noise.

The mice that were tested were the offspring of heterozygous parents. Due to this fact and the fact that the mice were not genotyped prior to testing, approximately one-quarter of the mice tested reported as mutant mice were actually wild-type mice. This means that the true effects of the mutations are likely considerably greater than those reported here. Further consideration needs to be given to a method of marking the young

pups so that a Southern blot can later be performed to confirm the genotype and this matched to the individual data records. Such a process would ensure that wild-type mice are not analyzed and reported as those of a mutant strain.

As outlined above, this study analyzed the response rate of mice between the age of 7 and 26 days old. Taking the health and well-being of the young mice into consideration, a decision was made to place the test chamber where the mice were individually tested over a heating pad during data collection. While the heating pad was placed under the cage in an effort to reduce the amount of stress on the young mice when removed from their litter, there is a possibility that the mice were made too comfortable in the sense that the test environment was not distinctive enough from their normal accommodations to elicit vocalizations, or "calls-for-help" to their mothers. Other researchers have found that when the mice are kept at a cooler temperature, better vocalization responses are obtained. Future researchers may wish to experiment with the degrees Celsius at which mice are tested when removed from their litters to find the temperature that elicits the greatest amount of vocalizations without causing distress to the test subjects.

Though vocalizations of mice removed from their litters of origin and placed alone in a test chamber were recorded and analyzed during this study, a future study may consider analyzing the response of an entire litter or perhaps the entire colony of mice. It would be interesting to compare the difference of call-rate when pups are removed from their mother and litter-mates versus when they are in their everyday environment. A comparison of call-rate between litters of wild-type and mutant mice would also be fascinating.

Another potential arrangement that may elicit a different pattern involves dividing the test chamber into three sections and separating the litter of pups and their mother by an unknown male mouse. This would involve placing the pups in the far right chamber, the mother in the far left chamber, and the unknown male in the middle chamber. It has been found that vocalizations of the pups are greatly increased when in the presence of an unknown male versus when they are separated from their mother without the presence from an unknown third-party.

Other studies (Miko et al., 2008; Shearer, 2012) show that these mutations affect hearing as measured by the ABR. Our present data now show different patterns in the unconditioned vocalizations of mice with Eph/ephrin mutations. There is one pattern in total vocalizations (and activity) and a different pattern in the percent of recorded activity in the 40-70 kHz range. Effects could also be related to the behavior of the mothers. This is likely a useful animal model of complex interactions among genetics, early experience, hearing and vocalizations. Research in this area may eventually be used as a parallel to research in the area of human infant babble comparing the vocalizations of deaf infants and their normal- hearing peers.

Conclusions

In summary, the results of this study demonstrated that the Eph/ephrin genotype, known to affect hearing, also affects neonatal vocalizations. Further, while it was determined that the group of $EB3^{Null}$ mice emitted the most ultrasonic vocalizations, it was found that the group that created the most noise (scuffles), the $EB3^{lacZ}$ mice, was the group that emitted the least amount of ultrasonic vocalizations. Additional analyses of effect of age revealed that while the vocalizations of the wild type mice peaked between 17 and 20 days of age, the vocalizations of the overall mutant mice population peaked between 11 and 16 days of age and were much greater than the wild type mouse population.

Chapter 2: Review of Literature

As most all young animals use various strategies to communicate with their parents; murine mammals, or mice, also use certain tactics to communicate with their mother. The vocalizations mice use to communicate with their mother are termed "wriggling calls," or "yelps" (Ehret & Rieke, 2002). These vocalizations are used by mice to elicit maternal behavior such as grooming, nursing, or protecting (Ehret & Rieke, 2002). Pup calls are emitted by isolated mouse pups between postnatal day 3 and P13 (Smith and Sales, 1980; Haack *et al.*, 1983). The vocalizations elicit a search and retrieval of pups by the mother (Sewell, 1970; Smith & Sales, 1976; Haack *et al.*, 1983). These calls undoubtedly serve an important communicative function (Liu et al., 2003).

Some research suggests that the ultrasonic vocalizations created by the mouse pups are dependent upon the mother's genotype, and the number of times calls occur may possibly be a reflection of maternal responsiveness (D'Amato et al., 2005). It is also possible that the amount of maternal responsiveness is affected by the genetic make-up of the strain of mouse supporting the idea that vocalizations could expose "genetic contributions to behavior (Liu *et al*., 2003, p. 3412)" (Bell *et al.*, 1972; Roubertoux *et al.*, 1996). In fact, a number of studies have been performed throughout the years by Bell et. al (1972) and D'Udine & Robinson (1982) where the ultrasonic vocalizations of various strains of mice have been sampled and compared. These studies have shown that large differences in the vocalization patterns of mice of different genetic strains may only take a single gene to affect the vocalization patterns of the pups. (Bell et al., 1972; D'Udine et al., 1982)

Some research suggests that the ultrasonic vocalizations of mouse pups may provide clues to behavior in adult life. A number of researchers have collected data that

suggest that those mice with communicative and/or social deficits identified early in life are at a greater risk for developing neurodevelopmental and neuropsychiatric disorders later in life including autism spectrum disorders (Scattoni et al., 2009). It is entirely possible, in fact, that a mouse exhibit autism. Thus, "understanding the types and functions of ultrasonic vocalizations emitted by laboratory rodents may enable researchers and animal care personnel to use vocalizations as an indicator of an animal's behavior and affect (Portfors, 2007, p. 28)." Further, it has been found that the interaction between the genetic makeup of the young subject combined with the effect of their environment has a strong relationship with the emotionality of a subject as an adult (D'Amato et al., 2005).

Aside from understanding the types and functions of vocalizations, it may also be important to establish an understanding of the areas in the brain that are responsible for control of vocalizations. Literature by Uwe Jurgens (2002) outlines the anatomical positioning of the reticular formation and ventrolateral parabrachial region in the brain. He has suggested that activity in the neurons of the parabrachial region as well as the reticular formation occurs during the emission of vocalizations as a result of the fact that a portion of the reticular formation maintains contact with the phonatory motor nuclei. This is supported by the fact that the neurons in the parabrachial region begin to fire prior to the start of vocalization, thus establishing them as an essential component of vocal pattern emission. (Jurgens, 2002)

Jurgens also suggests that the periaqueductal grey region is very important for vocalization production as it may contain the vocal pattern generator, or system responsible for the production of various vocalizations. The periaqueductal grey region

is also very important as it connects to interneurons that project into relay stations such as the nucleus retroambiguous also found to be a very important area for vocalization production. This is seen in studies of macaques by Larson (1991) and Larson and Kistler (1984, 1986) that revealed a change in activity level of various cells within the periaqueductal grey during emission of vocalization. (Jurgens, 1998) Further, studies of many species including the rat, guinea pig, chimpanzee, bat, and cat analyzed what happened when the periaqueductal grey was electrically stimulated. During stimulation, the vocal emission of species-specific calls that would be naturally produced were emitted and observed. (Jurgens, 2002)

Though Jurgens established the importance of the periaqueductal grey region in the production of vocalization, he also mentioned the importance of the inferior colliculus for similar purposes. It has been found that the periaqueductal grey vocalization pathway travels through the inferior colliculus and also synapses there. Due to this synapse within the inferior colliculus, its importance in the production of vocalizations is also very important to the emission of vocalizations. (Jurgens, 2002)

Another seemingly important area for vocalization generation is the anterior cingulate cortex. A study performed in 1974 by Sutton et al. utilized rhesus monkeys that were trained to emit a very specific vocalization in order to obtain a treat. It was discovered that when the anterior cingulate cortex was removed, the animals were no longer able to emit the vocalization and thus complete the task. Interestingly, another study of squirrel monkeys by MacLean and Newman (1988) found that when the anterior cingulate cortex was removed, the isolation peeps, or calls to others in their group as a means to re-gain contact when separated, were reduced. This impairment supports the

idea that the anterior cingulate cortex is indeed a very important area for the emission of vocalizations. (Jurgens, 1998)

As interesting as vocal production in other species may be, the primary goal of any animal research is to determine how the findings can be related to the human species. This study barely scratches the surface of efforts to better understand how genetics affect the development of vocalizations in other mammals, let alone how these findings translate to humans. That being said, there continues to be much research on the development of infant vocalization. It has long been believed that deaf infants and their age-matched peers with normal hearing develop vocalizations, or "babble," at the same rate. Research completed by Oller and Eilers (1988), however, suggests otherwise. Their research suggests that contrary to popular belief, deaf infants do not develop meaningful babble until much later in life than their hearing peers. Oller and Eilers report that hearing infants begin meaningful babble between 6 and 10 months of age while deaf infants do not begin to exhibit meaningful vocalizations until approximately 11-25 months of age. The underlying cause of the delayed babbling is due to the lack of hearing ability that has been found to be so instrumental in the development of speech sounds that comprise complex babbling, also known as canonical babbling. (Oller & Eilers, 1988)

Canonical babbling has been established as the most advanced stage of the four stages of babbling defined by Oller and Eilers. Three other stages of babbling precede it including the most basic stage termed the phonation stage, the gooing stage, and the expansion stage. The phonation stage occurs in normal-hearing infants between 0 and 2 months and is characterized by the production of comfort sounds mostly resembling

vowels. The gooing stage incorporates the vowel sounds established during the previous stage with consonant sounds. This stage is common in normally-developing children around 2-3 months old. The third stage, termed the expansion stage, builds upon the previous two but adds experimentation with volume and vocal quality and occurs around 4-6 months of age. Finally, the canonical stage begins somewhere between 7 and 10 months of age for typically-hearing infants. The canonical stage includes the production of reduplicated sounds (ex: baba) and the assignment of meaning to these approximations. (Oller & Eilers, 1988)

It is crucial that one appreciate the differences between the four stages of babble as without completely understanding these differences, one may falsely conclude that children babbling at different levels have achieved the same developmental milestones. Thus, when babbling is generically grouped into one category and not divided into stages, it is easy to see where one would make the mistake of assuming a deaf child actually babbling at the level of the phonation stage is thought to have reached the same advanced stage as their normal hearing peers. This fact may give some explanation as to the former wide-spread belief that deaf and normal hearing infants develop babble at the same rate. However, the separation of stages now firmly established and adopted by many language development experts provides researchers with a more accurate way of determining development of babble and how the development differs in normal hearing versus deaf children. (Oller & Eilers, 1988)

Though Oller and Eilers have established that normal hearing infants reach the advanced stage of vocalizations much earlier than deaf children of the same age, a study performed by Bass-Ringdahl further extended this thought by evaluating the babble of

children with various degrees of hearing loss. This study found that while those with mild hearing loss do not babble as early or as much as normal hearing infants, they do display more vocalizations than children with severe hearing loss. (Bass-Ringdahl, 2010) This fact supports the thought that individuals who can more normally perceive speech signals, particularly consonant information, will babble more quickly and at a more advanced level than those who cannot (Oller & Eilers, 1988).

Text written by Easterbrooks and Baker (2002) supports the fact that hard of hearing and deaf children develop vocalizations at a different rate than their normal-hearing peers. Easterbrooks and Baker also adhere to the four stages of babbling described by Oller and Ellers which explains that while no significant differences between the babbling of deaf and normal hearing infants are seen in the phonation or gooing stage of vocal development, one begins to see minor differences in the babbling of these two groups of children beginning in the expansion stage. Admittedly, differences in babbling are minor at this stage, but differences become much more apparent during canonical babbling. During canonical babbling, consonant-vowel combinations are produced by hearing infants while the babbling of the deaf infant does not include such productions and babbling is found to decrease at this point. (Easterbrooks & Baker, 2002)

Easterbrooks and Baker further elaborate that while there is a sequence in which hard-of-hearing and deaf children develop language, the development will be delayed as a direct result of their amount of hearing loss. In their text, Easterbrooks and Baker set forth seven stages of receptive and expressive language development in normal hearing children. An outline of these stages follows.

The first stage of development, early prelinguistic, occurs between the ages of 0 and 6 months of age. The listener will attend to a speaker's voice and respond to voices with which they accustomed. Expressive language ability includes "cooing" and babbling of vowels as well as various vocalizations and cries to communicate their needs with their caregivers. (Easterbrooks & Baker, 2002)

The next stage termed the later prelinguistic occurs between 6 and 12 months of age. At this point, the child knows his/her name and turns when called. The child is able to recognize familiar words and associate them with corresponding objects and people. At this level, the child's babbling begins to include a greater number of consonants and word approximations that they will use to communicate instead of always utilizing cries as in the previous stage. (Easterbrooks & Baker, 2002)

Between 12 and 18 months the single sign/word stage of development appears. At this stage, the child is able to understand and respond to commands and utters their first words. Intonation will continue to develop as the child produces jargon mimicking adult-like language. (Easterbrooks & Baker, 2002)

The fourth stage of development termed early word combinations occurs when a child is able to point to items or pictures when named and carry-out basic commands. This stage occurs between 18 and 24 months. The normal-hearing child should at this point be able to make two-word sentences while their vocabulary acquisition increases each day. (Easterbrooks & Baker, 2002)

The following developmental stage, multiword combinations, occurs from 24-36 months. At this point, the vocabulary of the child increases exponentially as he may learn 2-4 words each day! Multi-word sentences can be used to converse about a topic

and used to communicate with caregivers. The child's receptive language at this stage allows children to understand two-step instructions and complex sentences. (Easterbrooks & Baker, 2002)

The sixth stage termed expanded grammar will appear when the normal-hearing child is between 3 and 4 years old and can fully understand complex language. They are able to form longer sentences and create complex questions. Correct grammar becomes the norm, and the child is soon able to speak in abstract terms about events that have yet to occur. (Easterbrooks & Baker, 2002)

The final stage, adult-like language development, occurs at 5 years. Sentences including great amounts of detail are created which can be used to create lengthy fantastical stories. At this time, the child has developed a syntactic system that matches their large vocabulary. (Easterbrooks & Baker, 2002)

While the seven stages outlined above are a guide for the development of language in children with normal hearing, their deaf or hard-of-hearing peers will not reach these stages at the same time. Instead, children with hearing loss will develop vocalizations more slowly due to the inability to receive auditory feedback secondary to their damaged auditory system. (Easterbrooks & Baker, 2002)

Interestingly, though vocal babbling does not occur at the same rate in normal and hard-of-hearing or deaf infants, it has been found that infants with hearing impairment utilizing sign language for communication with their caregivers will "manually babble," or babble using signs instead of vocally-produced words. This manual babbling helps children using sign language to practice their signed communication strategies just as

hearing children use vocalizations to practice their spoken communication strategies. (Easterbrooks & Baker, 2002)

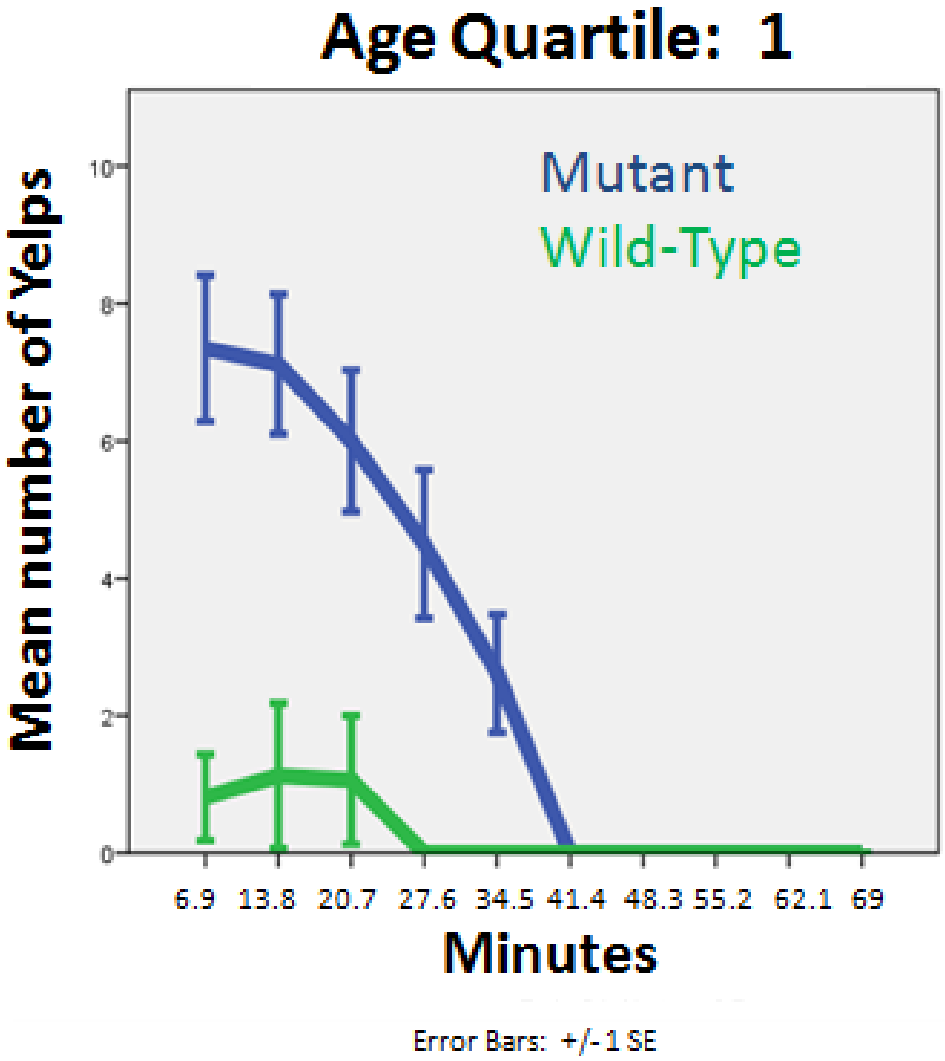
Though there is a great deal of information available regarding the development of speech and language in infants, there is still much to be learned. Despite the fact it has been well-established that hearing ability affects the development of infant vocalization, there remains a great deal to be learned regarding the effect of genetics on hearing ability and the resulting effects on speech and language development. With additional research in the area of genetics occurring every day, it stands to reason that the findings of such studies will have great impact on the services and treatment protocols recommended for individuals known to possess certain genetic predispositions in the future.

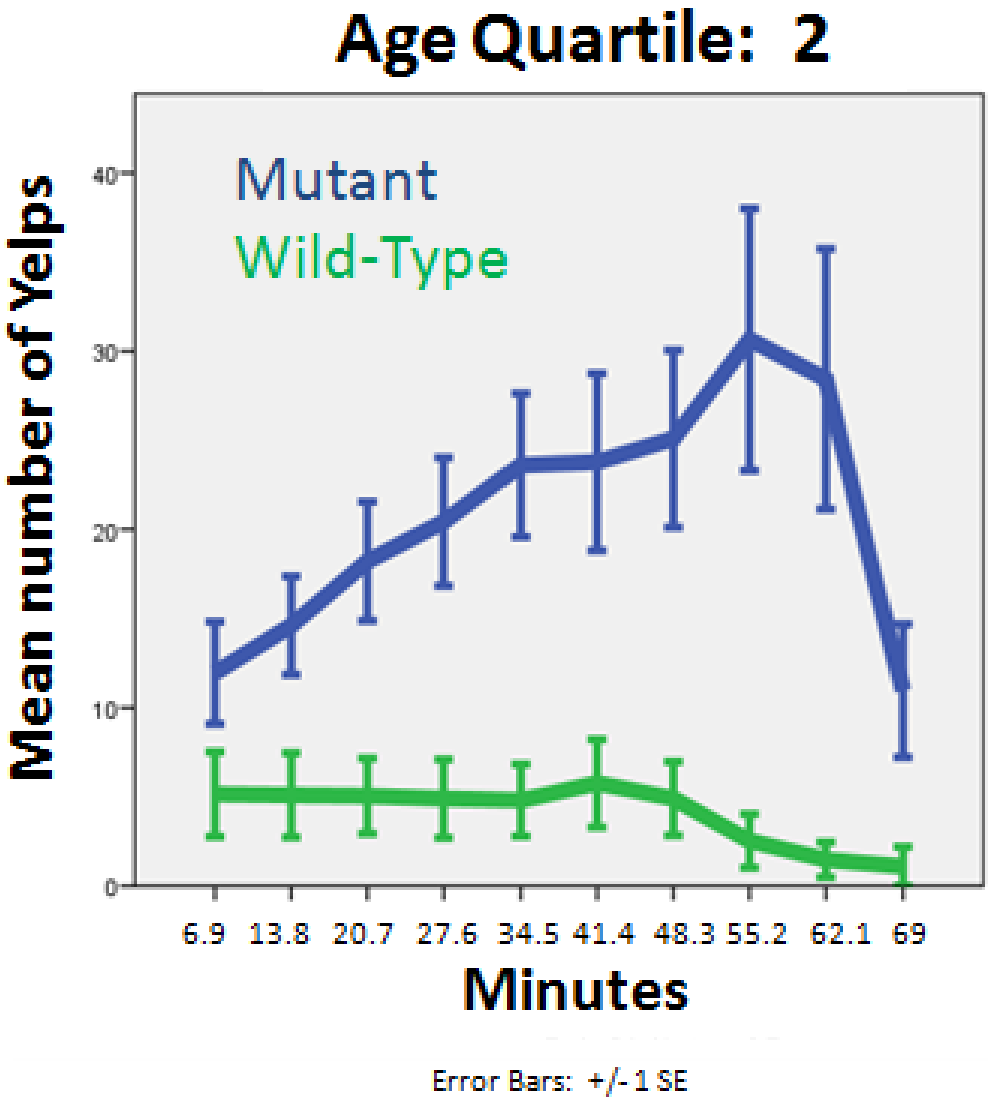
Chapter 3: Supplemental Data and Analyses

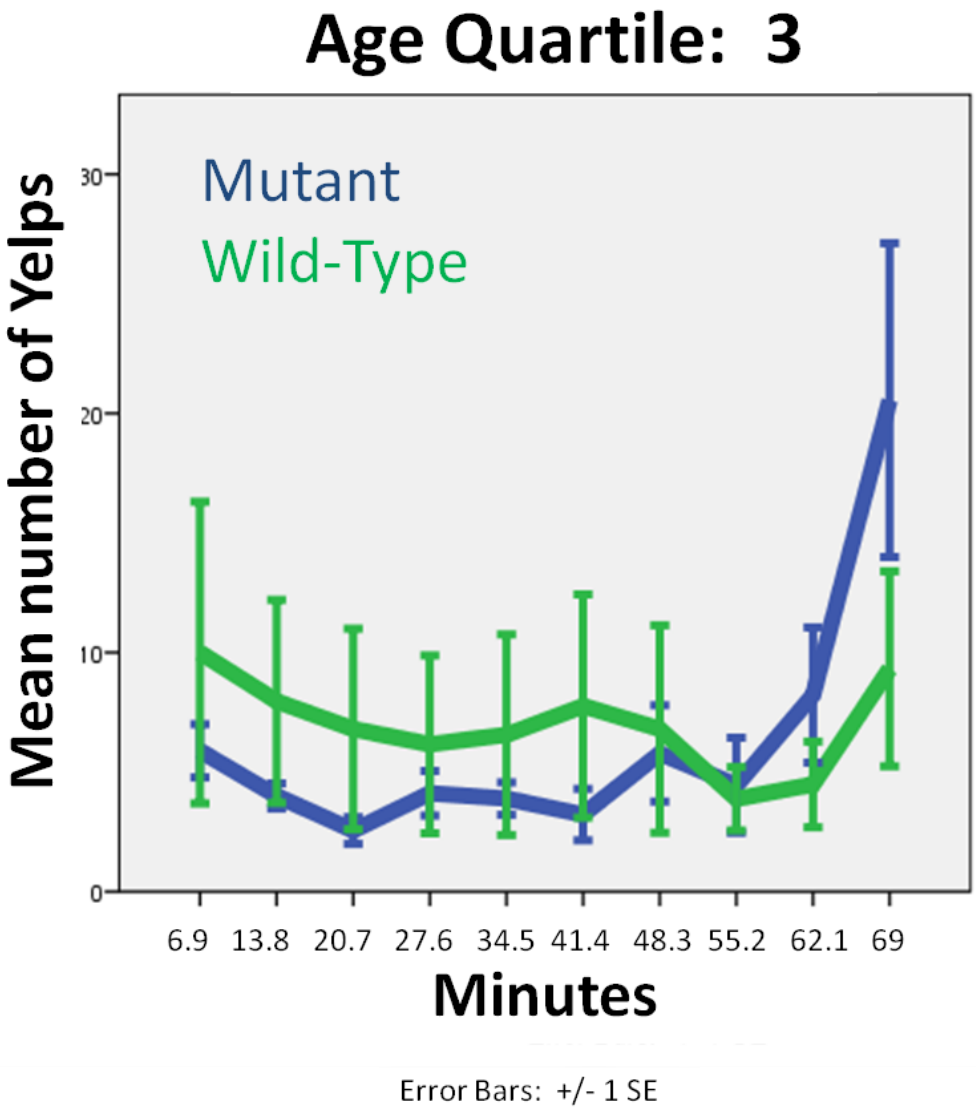
As mentioned in the above results section, analyses were also performed on the mice as divided by age quartile. As a review, age quartiles were determined by separating the test subjects into four equal categories as defined by their age: quartile one is comprised of the very youngest pup through subjects 10 days of age; quartile two is comprised of pups 11 to 16 days old; quartile three is comprised of pups 17 to 20 days of age; quartile four is comprised of pups 21 days of age to the very oldest mouse tested. Below, additional graphs are included portraying the comparison of the vocalizations of the various groups of mice measured during the four age-determined groups. Plotted are the numbers of vocalizations as a function of the time during the test.

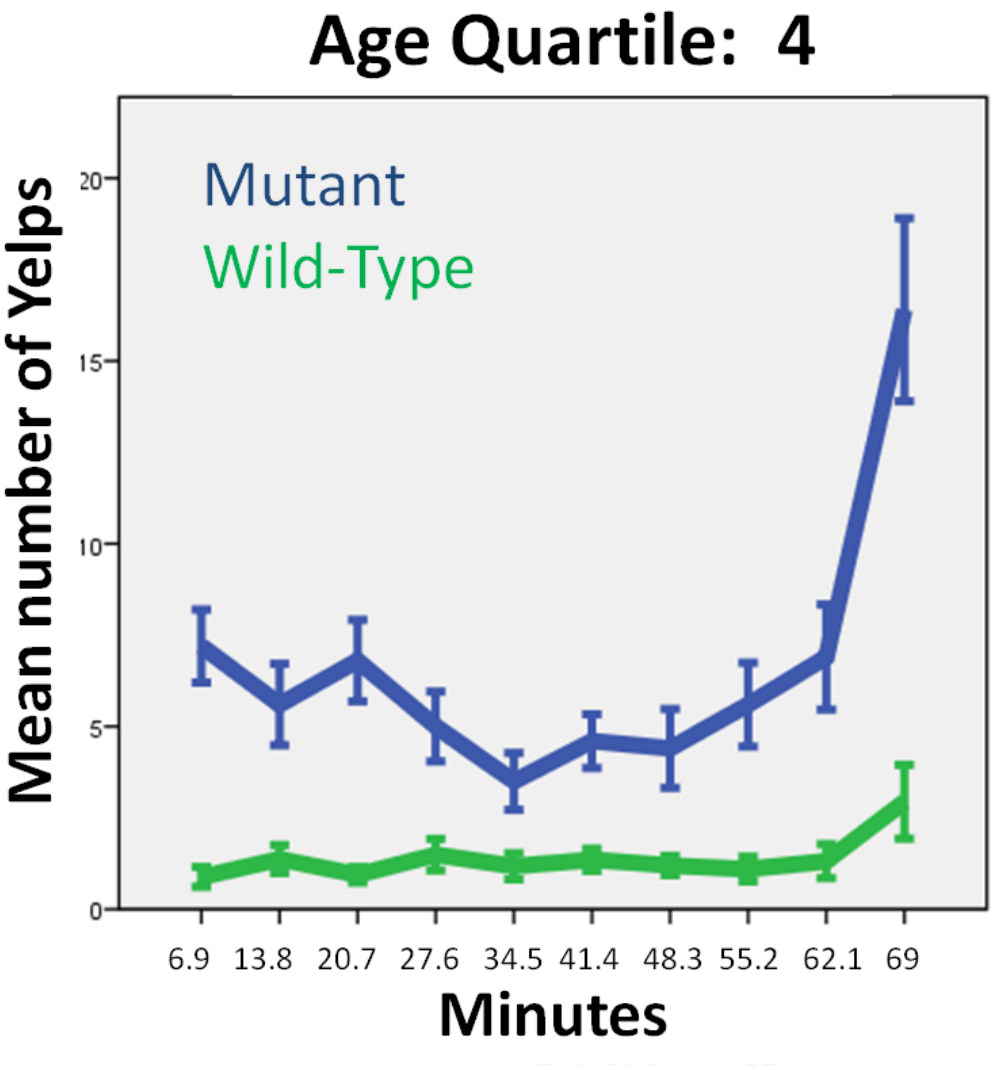
It is suggested in the graphs in Supplement A, below, that the mutant mice may have emitted more yelps as compared to the wild-type mice during all age quartiles except for during quartile 3 though the statistical significance to support this finding is still unclear. Interestingly, Supplement C shows that during age quartile 3 the wild-type mice emitted the most vocalizations, the only time they were found to create more emissions than the mutant mice. Supplement D clearly shows that it is during age quartile 2 that the mutant mice vocalize the most.

Supplement A: Time course of vocalizations over the duration of test in wild type mice as compared to mutant mice during various age quartiles. In the first quartile all yelps end after about 30 minutes because no pup was tested for a longer period.



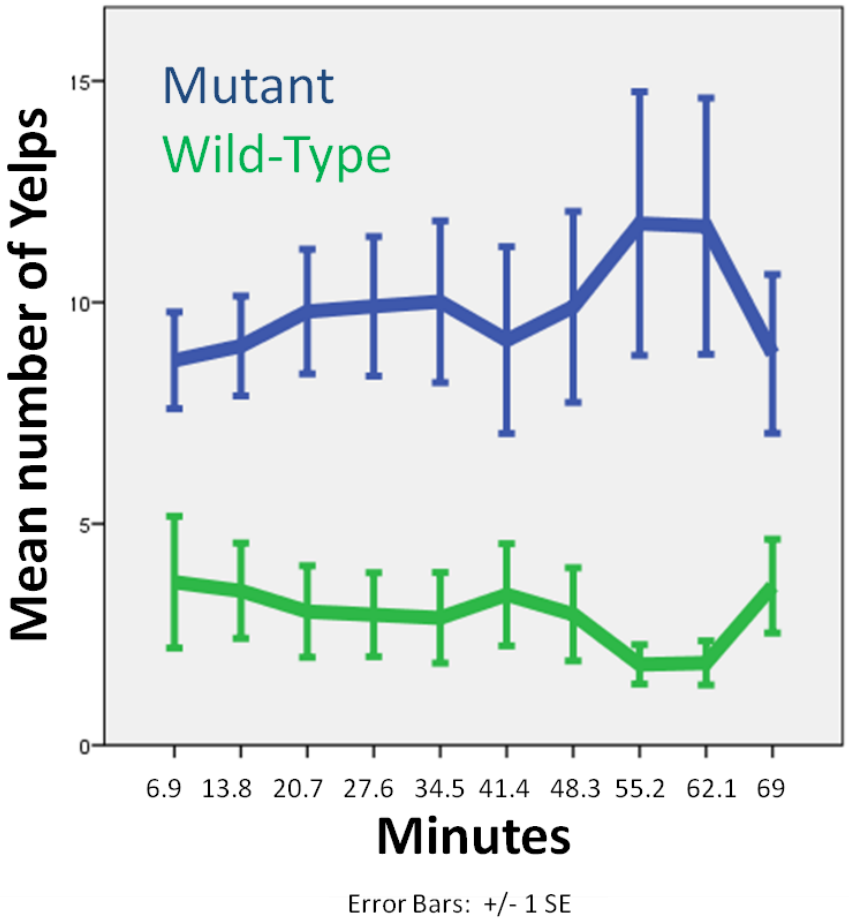

Age Quartile: 2
Mutant
Wild-Type
Mean number of Yelps
0
10
20
30
40
6.9
13.8
20.7
27.6
34.5
41.4
48.3
55.2
62.1
69
Minutes
Error Bars: +/- 1 SE


Age Quartile: 3
Mutant
Wild-Type
Mean number of Yelps
30
20
10
0
6.9
13.8
20.7
27.6
34.5
41.4
48.3
55.2
62.1
69
Minutes
Error Bars: +/- 1 SE

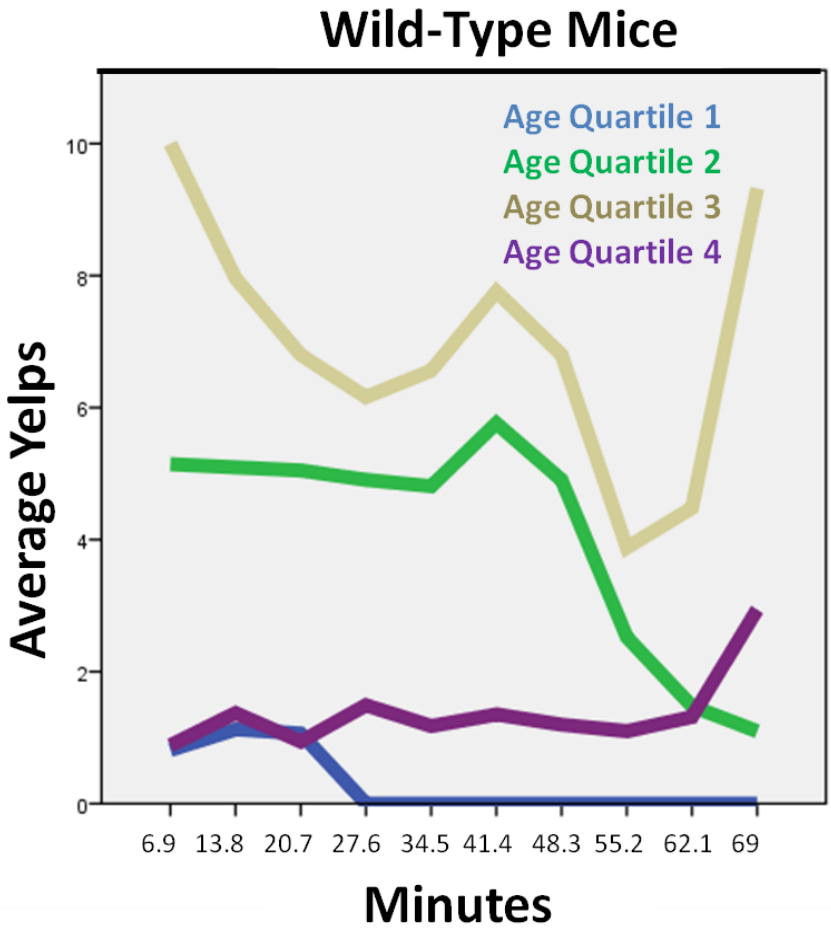


Error Bars: +/- 1 SE

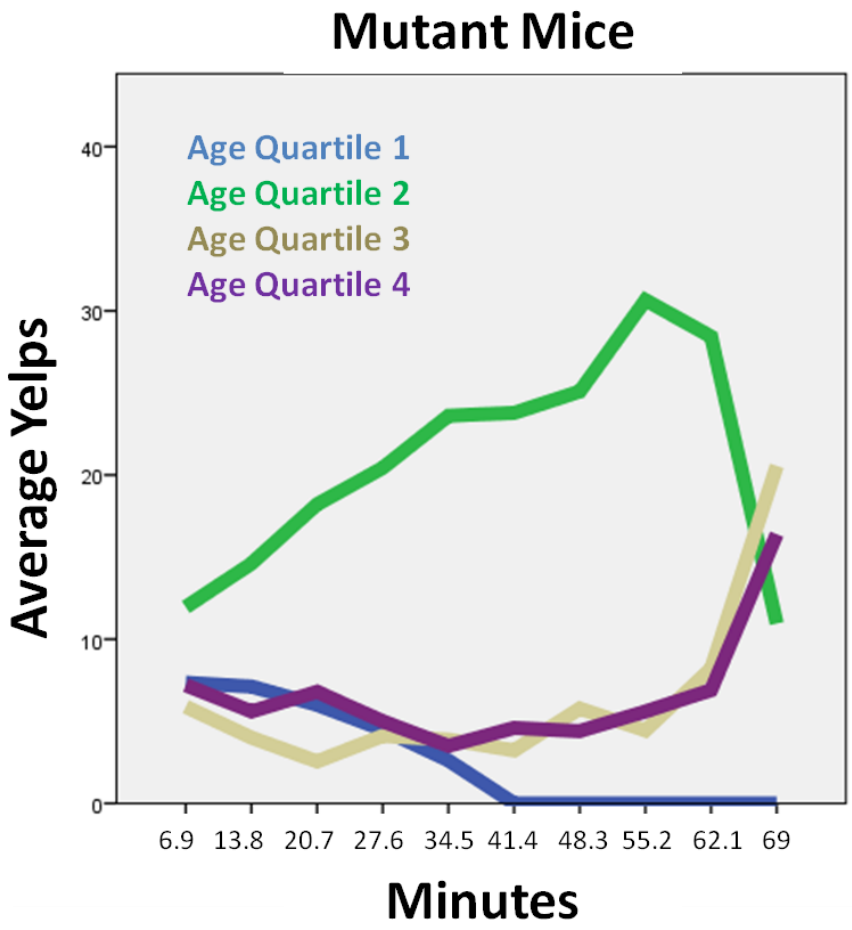
Supplement B: Yelps emitted by mutant mice as compared to yelps emitted by wild-type mice as a function of time. The mutant strain yelped significantly more than the wild-type strain throughout the entire duration of the test.



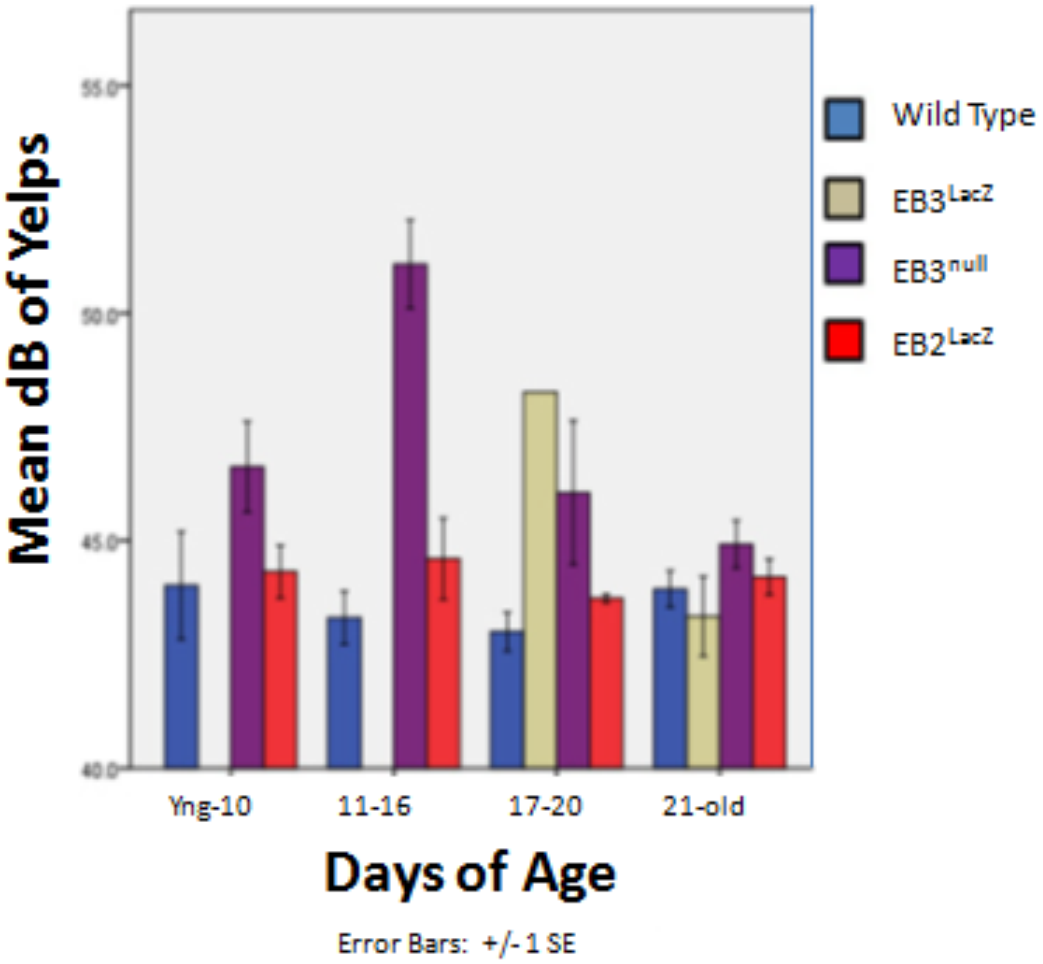
Supplement C: Time course of vocalization rate over the duration of tests in wild type mice of different age quartiles



Supplement D: Comparison of the vocalizations of mutant mice during different age quartiles

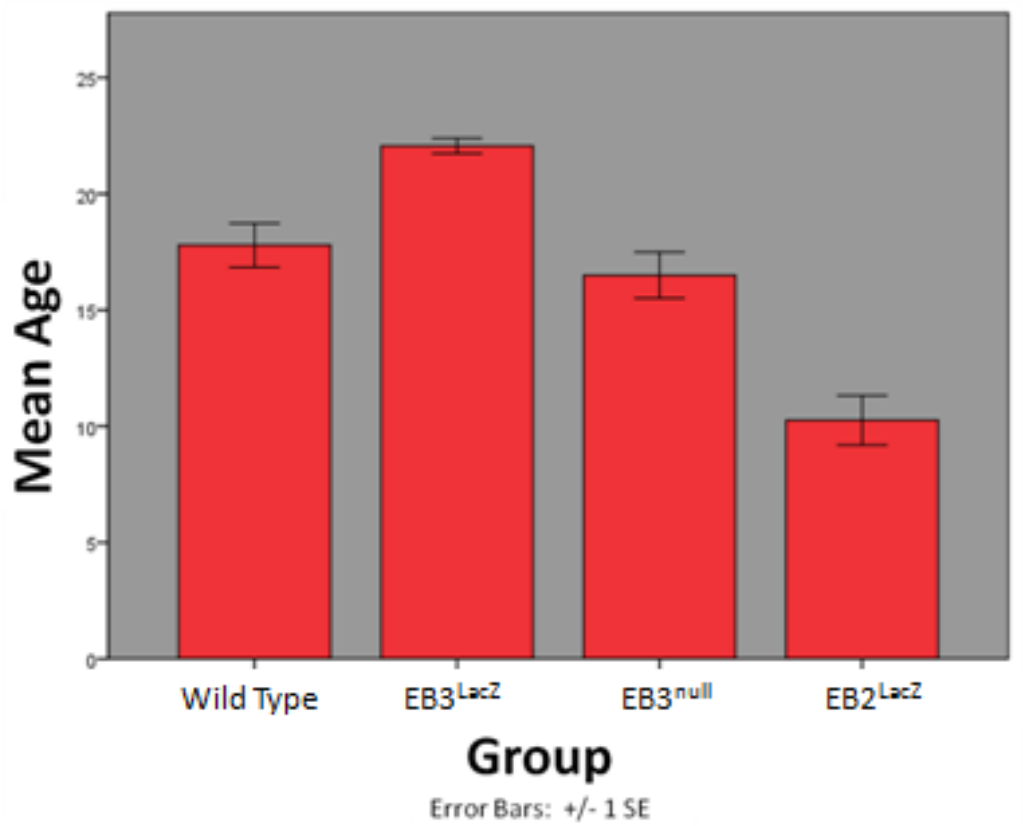


Supplement E: Comparison of the average loudness of vocalizations of all groups of mice at various ages



Supplement F: Average days of age of each group analyzed

This shows that though the wild-type mice and subjects in the EB3^{null} group were approximately the same age, the average age of the EB3^{lacZ} group was significantly older and the average age of the EB2^{lacZ} group was significantly younger.



Supplement G: Raw Data

This table includes all information found during data collection. Note; trigs denotes yelps + scuffles, or all activity recorded from each mouse.

Age	Group	Yelp/Hr	dBYelp	kHzYelp	Hours	Trigs	dBTrigs	kHzTrigs
19	ephrinB3lacZ	2	48	59	0.5	3	44	36
21	ephrinB3lacZ	4	42	60	0.5	4	43	47
21	ephrinB3lacZ	22	42	68	0.5	15	42	68
21	ephrinB3lacZ	2	40	66	0.5	2	40	48
21	ephrinB3lacZ	0		0	0.5	2	41	57
21	ephrinB3lacZ	4	44	66	0.5	4	43	39
22	ephrinB3lacZ	4	43	53	0.5	3	42	67
22	ephrinB3lacZ	4	42	73	0.5	5	42	65
22	ephrinB3lacZ	2	42	54	0.5	5	45	35
22	ephrinB3lacZ	4	53	67	0.5	5	47	69
22	ephrinB3lacZ	0		0	0.5	0		0
23	ephrinB3lacZ	2	44	66	0.5	5	46	70
23	ephrinB3lacZ	46	43	66	0.5	72	44	47
23	ephrinB3lacZ	4	42	67	0.5	12	44	63
24	ephrinB3lacZ	6	42	63	0.5	5	42	49
24	ephrinB3lacZ	0		0	0.5	8	42	60
24	ephrinB3lacZ	24	45	62	0.5	37	44	51
5	ephrinB3null	60	43	66	0.1	45	44	32
6	ephrinB3null	76	44	64	0.25	109	43	32
6	ephrinB3null	136	45	64	0.25	174	46	37
7	ephrinB3null	109	45	63	0.33	199	46	37
7	ephrinB3null	109	47	62	0.33	190	48	35
7	ephrinB3null	148	49	65	0.33	273	50	35
7	ephrinB3null	239	52	62	0.33	336	53	37
7	ephrinB3null	194	48	64	0.33	340	48	35
7	ephrinB3null	155	47	64	0.33	318	48	36
7	ephrinB3null	227	51	64	0.33	346	51	36
10	ephrinB3null	55	42	63	0.2	67	42	29
11	ephrinB3null	138	45	62	0.5	332	45	35
11	ephrinB3null	382	52	64	0.33	498	53	38
11	ephrinB3null	74	43	67	0.5	206	44	35
11	ephrinB3null	218	50	62	0.5	460	50	36
12	ephrinB3null	184	49	63	0.5	401	48	36
12	ephrinB3null	163	50	64	0.67	409	50	41
12	ephrinB3null	528	53	64	0.67	978	53	43
12	ephrinB3null	672	55	61	0.67	1075	55	45
13	ephrinB3null	112	48	63	0.67	471	46	31
14	ephrinB3null	628	56	60	0.75	1395	53	40
14	ephrinB3null	720	55	63	0.75	1488	54	44
14	ephrinB3null	724	56	62	0.75	1644	56	42
14	ephrinB3null	532	54	68	0.75	1181	53	44
14	ephrinB3null	677	55	64	0.75	1222	54	44
16	ephrinB3null	268	50	66	0.75	1047	47	33
16	ephrinB3null	67	45	66	1	790	45	26
16	ephrinB3null	391	51	65	0.75	972	49	38

17	ephrinB3null	55	43	63	0.33	200	43	25
19	ephrinB3null	44	43	67	1	307	42	31
20	ephrinB3null	61	44	64	1	645	44	27
20	ephrinB3null	50	42	63	1	479	44	27
20	ephrinB3null	179	51	63	1	898	49	34
20	ephrinB3null	10	49	67	0.5	37	47	50
20	ephrinB3null	24	51	67	0.5	28	51	55
21	ephrinB3null	86	44	64	1	768	44	30
21	ephrinB3null	8	46	60	0.5	19	44	47
21	ephrinB3null	14	43	66	0.5	19	43	51
21	ephrinB3null	50	49	63	0.5	75	49	48
21	ephrinB3null	6	42	75	0.5	9	42	72
21	ephrinB3null	26	44	64	0.5	31	47	49
23	ephrinB3null	4	44	67	0.5	5	47	43
23	ephrinB3null	10	42	64	0.5	20	44	58
23	ephrinB3null	36	49	62	0.5	36	50	67
23	ephrinB3null	74	46	66	0.5	172	46	52
23	ephrinB3null	26	48	67	0.5	48	48	50
23	ephrinB3null	4	43	70	0.5	15	44	50
23	ephrinB3null	28	48	69	0.5	43	48	61
25	ephrinB3null	50	45	64	1	635	44	26
25	ephrinB3null	69	44	64	1	875	45	25
25	ephrinB3null	59	45	63	1	1376	46	22
26	ephrinB3null	97	44	63	1	874	44	27
26	ephrinB3null	55	44	63	1	707	44	26
42	ephrinB3null	42	42	63	0.5	151	43	34
4	ephrinB2	40	43	62	0.1	31	42	31
4	ephrinB2	60	44	64	0.1	20	43	41
4	ephrinB2	210	47	66	0.1	78	48	38
5	ephrinB2	80	43	63	0.25	77	45	38
6	ephrinB2	128	48	63	0.25	137	49	34
6	ephrinB2	20	41	61	0.25	55	42	36
6	ephrinB2	68	46	61	0.25	124	44	31
6	ephrinB2	108	45	61	0.25	124	46	36
6	ephrinB2	116	48	62	0.25	129	48	36
7	ephrinB2	48	42	62	0.33	78	43	36
7	ephrinB2	48	45	68	0.33	78	43	34
7	ephrinB2	61	45	64	0.33	79	44	35
7	ephrinB2	30	45	64	0.33	64	44	41
7	ephrinB2	45	41	67	0.33	80	43	39
7	ephrinB2	58	42	62	0.33	112	43	33
11	ephrinB2	66	47	64	0.5	188	45	33
12	ephrinB2	54	42	66	0.67	311	43	28
12	ephrinB2	107	46	63	0.67	409	47	34
13	ephrinB2	176	47	64	0.67	500	47	37
13	ephrinB2	67	43	67	0.67	340	44	31
13	ephrinB2	46	42	63	0.67	239	43	32
18	ephrinB2	43	43	64	1	529	43	29
18	ephrinB2	54	44	65	1	398	43	32
18	ephrinB2	68	44	65	1	555	44	31
18	ephrinB2	47	44	65	1	492	44	29
21	ephrinB2	86	44	65	1	543	44	33
21	ephrinB2	57	45	66	1	511	45	29
6	WT	4	42	72	0.25	2	43	54
7	WT	0		0	0.25	0		0
7	WT	0		0	0.25	0		0
8	WT	115	46	67	0.165	58	50	56

8	WT	0		0	0.25	0		0
8	WT	4	40	65	0.25	2	43	42
8	WT	4	45	78	0.25	1	45	78
9	WT	0		0	0.25	0		0
9	WT	60	48	66	0.25	76	48	43
9	WT	40	47	65	0.25	38	46	45
10	WT	0		0	0.25	0		0
10	WT	3	40	50	0.33	1	40	50
10	WT	0		0	0.25	0		0
10	WT	0		0	0.25	0		0
10	WT	0		0	0.25	0		0
10	WT	0		0	0.25	0		0
11	WT	44	42	61	0.25	63	42	31
11	WT	0		0	0.35	0		0
11	WT	0		0	0.35	0		0
12	WT	60	43	63	0.3	96	43	33
12	WT	26	43	63	0.5	131	43	27
12	WT	142	45	68	0.5	298	44	41
13	WT	191	45	67	0.65	545	45	39
13	WT	218	46	68	0.65	528	45	41
14	WT	0		0	0.5	0		0
14	WT	20	40	77	0.05	20	44	30
14	WT	0		0	0.25	0		0
14	WT	474	42	65	0.5	421	43	51
14	WT	60	43	65	0.05	27	43	36
14	WT	0		0	0.25	0		0
14	WT	400	42	65	0.5	345	42	51
14	WT	60	46	63	0.1	53	44	28
15	WT	2	41	65	0.5	2	41	44
15	WT	0		0	0.5	0		0
16	WT	24	48	64	1	240	45	32
16	WT	0		0	0.5	0		0
16	WT	20	41	65	0.25	6	41	69
17	WT	0		0	0.5	2	41	33
17	WT	106	44	65	1	796	45	30
17	WT	2	41	63	1	6	42	61
17	WT	91	46	63	1	921	45	26
17	WT	0		0	1	2	48	70
17	WT	80	44	63	1	794	45	26
18	WT	1582	43	66	0.5	1129	43	57
18	WT	15	43	66	1	41	44	55
18	WT	86	45	64	0.5	338	45	28
18	WT	2	41	72	1	8	43	78
18	WT	3	40	76	0.33	1	40	76
18	WT	54	43	63	0.5	320	45	25
19	WT	22	43	64	1.5	123	43	51
19	WT	0		0	0.5	2	46	33
19	WT	10	46	65	0.5	15	45	59
19	WT	0		0	1	5	42	43
19	WT	82	44	63	0.5	483	47	24
20	WT	0		0	1	5	43	23
20	WT	370	41	66	1	518	41	58
20	WT	73	43	63	1	548	45	30
20	WT	1	41	65	1	4	41	49
20	WT	45	44	63	1	616	45	26
21	WT	17	42	64	1.5	87	43	52
21	WT	72	47	65	1	427	47	45

21	WT	4	42	72	1	27	43	50
22	WT	0		0	0.33	0		0
22	WT	1	40	76	0.75	2	40	45
23	WT	26	46	69	1	94	47	51
23	WT	1	47	63	1	13	47	54
23	WT	11	45	65	1	20	45	64
23	WT	29	45	66	0.75	77	45	53
23	WT	14	43	64	1	46	44	53
24	WT	0		0	1	0		0
24	WT	91	44	62	1	727	45	29
26	WT	0		0	0.5	0		0
27	WT	10	44	70	0.5	11	43	59
28	WT	43	44	63	1	132	45	54
28	WT	20	45	65	1.15	77	44	49
29	WT	25	44	61	1	63	45	50
33	WT	5	45	65	1	22	45	62
33	WT	15	43	65	1	70	43	46
40	WT	41	43	66	1	408	44	31
40	WT	68	43	65	1	443	44	32
58	WT	4	42	79	0.5	8	45	52
21	WT	80	44	64	1	754	45	26

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