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Characterization of ultrasonic hearing thresholds and vocalizations in the Dbh mutant-backcrossed CBA/CaJ mouse

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Abstract

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Vocal communication is an essential natural behavior that might be modulated by neuromodulatory mechanisms, such as the noradrenergic system. In order to study the effects of norepinephrine (NE) on the production of vocal cues and auditory processing, we generated a genetic knockout of NE by backcrossing a nonfunctioning dopamine beta hydroxylase (Dbh) allele from a C57BL/6J and 129/SvEv background onto CBA/CaJ mice. CBA/CaJ mice are known for better high frequency hearing, which allows us to study auditory processes that involve natural, socially relevant ultrasonic vocalizations. In this study, we characterized this genetic knockout in parameters such as hearing thresholds, including at high frequencies, and vocalization production during development. To characterize hearing thresholds we used auditory brainstem response recording and to characterize vocalization production we recorded isolation-induced pup ultrasonic vocalizations. Hearing thresholds and acoustic features of vocalizations produced by Dbh backcrossed mice, both heterozygotes and wild-types, did not differ significantly from the background CBA/CaJ mouse strain, and had significantly better hearing than the Dbh mutant background strain. Within the NE-competent Dbh backcrossed CBA/CaJ mice, we compared the acoustic features of the vocalizations from the Dbh +/+ wild-type mice with Dbh +/- heterozygotes and found no differences in vocalization frequencies, durations, and rate of calling. Hearing was not different in NE-competent CBADbh mutants

compared to CBA/CaJ mice. These results indicate that a single mutant Dbh allele does not affect normal vocalization production and hearing. In one Dbh knockout (-/-) mouse, we observed decreased rates of vocalizations, suggesting NE knockout mice might have abnormal vocal production.

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Introduction

Vocal communication is one of the primary ways to interact with others. It allows us a means to convey ourselves via oral production of sounds, and to perceive what others are trying to communicate by listening to them. Since vocal communication is one of the primary senses of hearing-capable humans, dysfunctions in this system would lead to a decline in quality of life. It is not surprising that there is a great deal of interest in the study of vocal communication, which can be broken down into two main aspects: vocal production and audition.

To understand the basic neural mechanisms involved in vocal communication, we have to take into consideration the roles of neurotransmitter systems and neuromodulatory systems. It is known that neuromodulators are implicated in the modulation of behavioral states, which in turn might modulate the auditory cortical responses to sound stimuli (Gu, 2002). Dysfunctions of neuromodulatory pathways are associated with diseases affecting sound perception, such as tinnitus and schizophrenia (Yamamoto and Hornykiewicz, 2004, Manzoor et al., 2013, Coomber et al., 2015). As a result, there is a rising interest in the mechanisms of auditory cortical plasticity. One neuromodulator in particular, norepinephrine (NE) levels has been correlated with symptoms of schizophrenia (Yamamoto and Hornykiewicz, 2004). Research done on the songbird model has shed some light on the involvement of NE in both vocal production and auditory perception. One such songbird study found that a noradrenergic neurotoxin treatment significantly decreases both the quantity and quality of courtship singing in males, biasing female songbirds toward control males that were not drug treated in a mate choice test (Barclay et al., 1996). Furthermore, noradrenergic neurotoxin-treated females do not distinguish between high quality or low quality male courtship songs, and thus spend equal amounts of time with

both treated and control male songbirds during a mate choice test (Vahaba et al., 2013). These findings in songbirds strongly suggest NE involvement in vocal production and auditory perception. However, these results were not yet shown in a mammalian model, which would have better validity for potential translational research in the future.

In studying vocal communication, the mouse is a well-established and popular mammalian model because of its many benefits: the anatomical and functional similarities of the mouse central auditory system with other mammals make it an economical yet invaluable model to study neurobiological mechanisms underlying vocal communication (Zheng et al., 1999, Liu, 2006, Ohlemiller, 2006). The species' breeding capacity, short life span, ease of handling, and overall relatively cheap costs associated with animal research makes it an especially attractive animal model to be used for genetic manipulations. Knockouts of targeted genes are a valuable tool because it could elucidate the specific roles of a gene product in vocal communication. In studying vocalization, pup isolation calls have been well studied in mice since their first discovery (Zippelius and Schleidt, 1956, Sewell, 1970). Isolation calls are ultrasonic vocalizations (USVs) with a frequency range typically between 30 kHz and 90 kHz, which elicit a maternal response where mothers will retrieve pups back to the nest (Ehret, 2005). These ultrasonic vocalizations are distinguishable from adult encounter calls via their spectral and temporal properties (Liu et al., 2003), and these vocalizations carry differentially salient cues to the receiver (Shepard et al., 2015b). These findings confirm that vocalizations in mice do contain communicative properties. Furthermore, experienced mothers and naïve mice perceive pup calls differently. Specifically, maternal mice exhibit enhanced neural discrimination towards salient pup calls when compared with the nonmaternal response (Shepard et al., 2015b). Mouse vocal production and sound perception has been previously established, but NE has not yet been well

studied in mouse vocal communication and not much is known if it serves a modulatory role in these processes.

To study the role of NE in mouse vocal production and auditory processes, the dopamine beta hydroxylase (Dbh) knockout mouse is a good animal model. These mice lack the enzyme DBH that is responsible for converting dopamine to NE, and are thus incapable of producing NE (Eggermont and Roberts, 2004). Using these mice, our lab has found that NE is necessary for developmental tonotopic map expansion (Shepard et al., 2015a).Tonotopic map expansion following passive sound exposure in normal developing mice is an example of neuroplasticity, as the local brain area dedicated to the sound stimuli have an increase in map representation following exposure. However, map plasticity has only been documented in laboratory conditioning paradigms (Weinberger, 2004), and is not evident in a social learning paradigm such as the maternal response to pup calls. Since map plasticity is not seen in maternal mice that are experienced with high frequency pup calls, plastic processes other than cortical map plasticity, such as firing rate of putative pyramidal neurons, must encode the information necessary to recognize perceptually salient vocalizations (Shepard et al., 2015b). In order to effectively study the role of NE in auditory cortical plasticity implicated in maternal learning of high frequency pup calls, we backcrossed the Dbh mutant gene from the mixed C57BL/6J and 129/SvEv background, which has relatively poor high-frequency hearing, onto a CBA/CaJ background, which is a better high-frequency hearing strain (Willott, 1986). Since our goal was to study auditory cortical responses in postweaning mouse mothers, age-related hearing loss is another factor that should be considered as mothers might start to show signs of hearing loss in the study period. To further establish the advantages of the Dbh-backcrossed CBA mouse model, henceforth noted as CBADbh, these animals show significantly less age-related hearing loss than

the C57BL/6J strain (Walton et al., 1995). In addition to studying the role of NE in auditory cortical plasticity, we were interested in the involvement of NE in mouse vocal production, as motivated by the prior songbird studies.

We hypothesized that USV features between CBADbh+/- and CBADbh+/+ are the same, due to previous findings that Dbh+/- mice that were heterozygous for the Dbh mutation had NE levels indistinguishable from Dbh+/+ wild-type mice (Thomas and Palmiter, 1997b, Thomas et al., 1998). To ascertain whether the backcrossing was successful in generating a mouse strain that allows for manipulations of NE, and exhibit high frequency hearing, the hearing thresholds of CBADbh mice were evaluated by recording their auditory brainstem response (ABR). The ABR is a common and reliable metric used for assessing hearing in mice by measuring the electrical activity of the early auditory processing pathways (Johnson and Brown, 2005, Zhou et al., 2006). ABR recordings consist of five peaks, which represent in order, the auditory nerve, cochlear nucleus, superior olivary nucleus, lateral lemniscus, and inferior colliculus (Henry, 1979, Ruth and Lambert, 1991).

Materials and Methods

Animals

All procedures were approved by the Emory University Institutional Animal Care and Use Committee. First generation CBADbh animals were produced by mating homozygous Dbh-knockout male mice on a mixed *C57BL6/J* and *129SvEv* background with female CBA/CaJ mice. Subsequent backcrossing was done by mating heterozygous Dbh mutant offspring with CBA/CaJ mice until the 8^{th} generation of CBADbh mice was produced. Animals in the study were offspring from inbreeding of the 8^{th} generation CBADbh mice (Fig. 1). Female mice set up for mating with a male mouse were checked daily for vaginal plugs. Nine days after mating has occurred, at embryonic day 9.5 (E9.5) to E14.5, pregnant mothers with potential Dbh-/- offspring had their drinking waters replaced with a water solution mixed with alpha and beta adrenergic receptor agonists, phenylephrine and isoproterenol respectively (0.02M ascorbic acid, 0.01M phenylephrine, 0.01M isoproterenol). At E14.5, L-threo-3,4-dihydroxypheylserine (DOPS) water solution, a NE precursor, was given (0.01M ascorbic acid, 0.01M DOPS) until parturition. Drug treatments that serve to replenish NE levels during Dbh-/- embryo development were necessary for the survival of these pups in utero. Pups were approved for extended weaning of 28 days and were then housed in groups of not more than 5 with their same-sex littermates under a normal light cycle (lights on 7AM – 7PM). All animals had access to food and water *ad libitum*. Animals were aged between postpartum day 5 (P5) and day 9 (P9) for vocalization recordings and 14-20 weeks old at the time of auditory brain stem recording.

Identification tagging of mouse pups

Mouse pups had their toes tattooed with sterile ink (StarBrite Lime Green Sterile Tattoo Ink) at postpartum day 3. Sterile needles (27 ga x ½ in PrecisionGlide Needle, BD inc., Franklin Lakes, NJ) were used with a syringe (Becton Dickinson 1-mL syringe) to draw and inject tattoo ink into the dermis layer of the toe. Secondary tagging on the pup tail was done as a precautionary measure to prevent confusion and possible mix-up if the toe tattoo fades. Bright and non-Red tattoo colors were used to avoid color fading and prevent distress to the pup mother.

PCR genotyping of animals

Mouse tail snips (<5 mm) were collected from tattoo-tagged P3 pups using a pair of sanitized sharp scissors. Tail snips were placed into labeled individual 1.5 mL eppendorf tubes and flash frozen with liquid nitrogen. The eppendorf tubes were dunked in liquid nitrogen until boiling stops, and were then stored in a freezer until ready for genotyping. All mice were genotyped by PCR by the Weinshenker lab. The two pairs of PCR primer sequences used to amplify portions of the Dbh locus were the same as previous publications (Thomas et al., 1998).

Isolation-induced pup ultrasonic vocalizations recording

Pup isolation calls were elicited by removing a pup from its nest and placed in a clean cage setup within an anechoic chamber. A microphone (Brüel & Kjær, Nærum, Denmark) is suspended above the clean cage to record pup vocalizations at a sampling rate of 223214.2857

sample/s. Individual pups were tracked according to the pups tattoo and its isolation calls were recorded at P5, P7, and P9. Each recording lasted 10 minutes for individual pups.

Detection of pup ultrasonic vocalizations

Recordings were high-pass filtered at 25 kHz using 8-order Butterworth filtering MATLAB software (MATLAB, The MathWorks, Natick, MA, USA) to attenuate low frequency noise. Technical details of the pup call detection algorithm using computer algorithm were previously published (Liu et al., 2003). In order to create spectrograms (Fig. 2a,b) for visual representations of audio recording, the native .f32 files were required to be converted into a .WAV file using the MATLAB wavwrite function. A background noise region was manually identified (spectrogram visualized with Audacity Sound Editor 2.0.6) for each audio file and its start and end (in sample points) were inputted into a file to be used for noise reduction. Recording sound files were denoised using the "spectral subtraction" algorithm in MATLAB which subtracts the background noise of each recording (Boll, 1979). Following denoising, it is possible to implement algorithms that will automatically detect and list possible mouse vocalizations within the 10 minute recording. In order to check for consistency and reliability of the algorithms, a hundred random pup calls that were detected were cross-checked with a spectrogram. The pup call detection algorithm outputs vocal details such as duration, frequency, amplitude, rate of change in call frequency, and bandwidth of calls.

Visual representation of vocal characteristics overlap in CBA and CBADbh mice

To compare with previously published data of CBA pup vocalizations, probability distribution clouds of frequency and duration for CBA and CBADbh mice (+/- and +/+ groups

were combined) were each graphed on a contour map using a custom-made MATLAB code that was previously used in the lab to represent similar data in past publications (Liu et al., 2003, Shepard et al., 2015b). Contour maps were generated for pooled P5, P7, and P9 vocalizations, and also separately generated for each P5, P7, and P9 vocalizations of the CBA and CBADbh mice. The contour maps overlaid on top of each other using Adobe Photoshop CS6 (Adobe Systems Inc., Mountain View, CA) for a visual representation of the overlap in vocal characteristics in these two mouse strains.

Comparison of vocalization frequencies in CBA, CBADbh+/-, and CBADbh+/+ mice

Every instance of vocalization across ages P5, P7, and P9 in the CBA, CBADbh+/-, and CBADbh+/+ mice groups was plotted as points on the scatter plot by their mean frequency (kHz). Through visual inspection, clusters of the vocalization frequencies were grouped arbitrarily into high (> 85 kHz) or low (< 85 kHz) frequency ultrasonic vocalizations. Cumulative distribution function (CDF) was also plotted as another way to validate the effectiveness of an 85 kHz threshold.

Comparison of vocal characteristics averaged within individual mice

Average mean frequency for an individual pup's calls in a 5-minute recording period was separately averaged according to low or high frequency groups as previously classified (Threshold of 85 kHz for high frequency), and was grouped by the age at which recording occured. Each of these data points represents an animal's mean frequency when vocalizing in either low or high frequency calls for each day. Duration for an individual pup's calls were

similarly averaged and grouped by age, but without the distinction of short or long calls classification because the durations of calls did not show an apparent clustering.

Comparison of pup vocalization rate in CBADbh+/-, CBADbh+/+, and CBADbh-/- mice

Vocalization rates (calls/s) were defined as the number of calls in a 5-minute period divided by 300 seconds. Call rates were determined for individual animals at each day of recording and were then averaged by their respective genotypes to yield a group average call rate at each day.

Induction and maintenance of anesthesia

Animals were anesthetized with an intraperitoneal (IP) injection of ketamine (100mg/kg) and xylazine (5mg/kg), and allowed 10 minutes for induction of anesthesia in an anechoic chamber where recording or surgical procedures will take place. After sufficient induction of anesthesia, the animal is transferred onto a heated pad that adjusts temperature levels to maintain the core temperature of the mouse at 37 °C, as measured by a rectal temperature probe (DC Temperature Controller, FHC Inc., Bowdoin, ME). Live video feed of the anesthetized animal is set up for the experimenter outside the chamber to observe the animal's condition during recording. For longer procedures requiring secondary doses of anesthesia, an IP cannula was inserted immediately after the induction of anesthesia. The IP cannula is prepared by threading polyethylene tubing (PE10 tube of 0.011" x 0.024" inside diameter by outside diameter, VWR Scientific inc., Radnor, PA) through a needle (20 ga x 1 ½" PrecisionGlide needle, BD inc. Franklin Lakes, NJ) to be inserted intraperitoneally. The other end of the PE tube is connected to a needle syringe containing ketamine (30mg/kg) and xylazine (1mg/kg) to be used for

maintenance of anesthesia. Maintenance doses were given when animals were responsive to the toe-pinch reflex.

Auditory brainstem responses

To ensure hearing thresholds of the CBADbh animals are comparable to the parent CBA/CaJ mice, hearing thresholds of both WT and heterozygous CBADbh mice were assessed by recording auditory brainstem responses (ABRs). CBADbh-knockout (KO) mice will also have their ABR measured to confirm that their hearing is not affected by the mutant Dbh gene. Animals were anesthetized with a single dose of ketamine/xylazine mix that will usually last the whole procedure. If an animal shows signs of waking from anesthesia before the ABR session ends, the procedure is ended immediately with the animal placed back into its home cage for recovery. Secondary injections of ketamine/xylazine were avoided because additional anesthetic agents or the deeper level of anesthesia might affect the hearing thresholds.

Setup and procedures used for the ABRs were described in a published study from our lab (Miranda et al. 2014). Click stimuli and pure tones at 8 kHz, 16 kHz, 24 kHz, 32 kHz, 64 kHz, and 80 kHz were played from a speaker (HiVi RT1.3 Planar Isodynamic Tweeter) positioned 11 cm away from the right ear. Using Tucker Davis Technologies BioSigRP © software along with its System 3 hardware, ABR signals were sampled at 24 kilosamples/s using needle electrodes inserted subdermally into the vertex of the skull and each tympanic bulla. The active lead was placed at the vertex of the skull, with the ground electrode overlying the left bulla and reference electrode overlying the right bulla. Click stimuli and tone stimuli at each frequency were presented 500 times to acquire an averaged ABR waveform for the corresponding stimulus. For each stimulus, sound intensities were decreased in 5dB intervals,

starting at 78 dB SPL. Intensity was reduced until no sound-evoked peaks were apparent in the averaged waveform. Hearing thresholds were determined as the lowest intensity at which sound-evoked peaks could be seen (Fig. 3).

Auditory cortical tonotopy - electrophysiological mapping

Tonotopic organization of the auditory cortex in CBADbh mice were assessed by electrophysiological mapping of the auditory cortex after a successful ABR. Prior to mapping, a craniotomy is needed to expose the auditory cortex. Animals were secured on a bite bar of a stereotax (Model 900, David KopfInstruments, Tujunga, CA) and oxygen was supplemented through a tube attached to the nose clamp of the bite bar. Fur on top of the skull was trimmed and removed with Nair hair remover lotion (Church & Dwight Co Inc., Ewing, NJ). Following hair removal, an incision was made down the midline of the scalp. Four Schwartz vessel clips (World Precision Instruments Inc., Sarasota, FL) were clipped at the four corners of the incision to keep the incision open to reveal the skull. A periosteal elevator was used to pry away muscle connecting the skull and the skin. The left temporal muscle was detached from the skull and excess portions were cut with scissors until the zygomatic arch was visible. Figure 4a,b.

The boundaries of the craniotomy were identified with anatomical landmarks and then marked with a non-toxic marker. The rostral boundary was 30% the distance from bregma to lambda, while the caudal boundary was 90% of the distance. The ventral boundary was above the zygomatic arch, while the dorsal boundary was placed such that a square area (3mm x 3mm) would be enclosed by these boundaries (Fig. 3a). Typically the dorsal boundary is about 1mm dorsal to the ridge formed between the dorsal and ventral surfaces of the mouse skull. After defining the boundaries, a headpost (Inverted flat-head machine screw, 0.19” head diameter x

0.47” length) is secured with dental cement (Maxcem, Kerr, Orange, CA) at the dorsal surface of the skull immediately behind bregma. A small screw (Flat-head machine screw, 0.054” head diameter x 0.09” length) was driven into the skull and connected to an electrically grounded stainless steel wire loop.

The animals were removed from the stereotax and secured onto a mount via headpost for the craniotomy. The craniotomy was performed with a #1/4 carbide burr (CircuitMedic, Haverhill, MA) attached to a dental drill (OmniDrill35, WorldPrecisionInstruments Inc., Sarasota, FL) Immediately after removing the skull, a drop of silicon oil was applied to the exposed cortex to prevent drying. A high-resolution photo of the cortical surface is taken under the microscope to be used for planning and keeping track of electrode insertion points (Fig.3b).

For electrophysiological recording, a micromanipulator with hydraulic drive (FHC Inc., Bowdoin, ME) was used to penetrate 4MΩ 3x1 tungsten matrix microelectrodes (FHC Inc., Bowdoin, ME) into the cortex at a depth of ~400 µM, layer IV of the auditory cortex. Tones of varying frequency (log-spaced frequencies from 5 kHz to 90 kHz) were played from the speaker setup previously described in the ABR section. The frequency which elicits the highest firing rate of the cortical area were marked on the high-resolution photo of the cortex as the best frequency (BF) for that penetration.

Statistical analysis

All data were analyzed using JMP Pro 10 (SAS Institute 2012, Cary, NC). Mean frequency (kHz) of all pup vocalizations were analyzed using the non-parametric Kruskal-Wallis test due to non-normal distribution of data among three groups. Because of the significant clustering of data, all subsequent frequency analyses were analyzed according to low or high

frequency groups previously defined. In the comparisons of vocal characteristics, data were averaged within individual animals. Averaged vocalization frequencies and durations for the groups were analyzed *post-hoc* using the Steel-Dwass method for multiple comparisons of non-parametric data. For the call rate comparison between CBADBh+/- and CBADbh+/+, Wilcoxon rank-sum test was used since there were only two groups.

The hearing of mice in response to click or tone stimuli was transformed into a nominal variable of hearing or non-hearing. The proportions of hearing to each stimulus were analyzed using Pearson's Chi-Square analyses to show the presence of significant group differences. Hearing thresholds of the ABR stimuli were analyzed for the three groups using Steel-Dwass method to correct for multiple comparisons.

Results

We first qualitatively assessed the pup ultrasonic vocalizations of CBA and CBADbh pups to confirm no obvious alterations in basic vocalization properties as a result of Dbh-mutant gene backcrossing. Call properties were also quantitatively assessed to test the hypothesis that USV features between CBA pups and the NE-competent CBADbh pups, CBADbh+/- and CBADbh+/+, are the same. To quantitatively measure hearing capabilities in the newly generated CBADbh mice, hearing thresholds of the NE-competent CBADbh mice were evaluated by recording their auditory brainstem response (ABR). Data for one CBADbh-knockout mouse was also included whenever possible to show comparisons between the NE-incompetent mouse and the NE-competent mice.

Qualitative comparison of CBA and CBADbh pup vocalizations

Because of an interest in testing the similarities between CBA and CBADbh vocal properties, probability density clouds of call duration by call frequency were constructed for CBADbh and CBA vocalizations (Fig. 5a). Similarities in basic vocalization properties can then be visualized from the contour maps of these clouds, where the density of the cloud is correlated with the probability of a call at that frequency and duration. The overall vocalizations, taken by pooling all vocalizations in P5, P7, and P9, shows a clear overlap of the contour maps for pup vocalizations of these two mouse strains. Previous publications comparing call clouds between CBA pup isolation calls and CBA adult encounter calls had a lot less overlap in their call duration and frequency (Liu et al., 2003, Shepard et al., 2015b). This lack of overlap between adult and pup calls suggests that basic properties of calls that are inherently different can be

distinguishable when visualized with a contour map. Therefore, the overlap between contour maps of CBA and CBADbh pup vocalizations suggests that isolation induced pup ultrasonic calls are similar for these two strains. Although interestingly, there appears to be a small number of high frequency calls that had longer durations in the CBADbh pups. To better picture the development of pup vocal properties, probability call clouds for each day, P5, P7, and P9 were also constructed to visualize possible changes in call characteristics over time (Fig. 5b,c,d). Separating these call clouds into each day, the lower frequency vocalizations show considerable overlap, but the longer duration of CBADbh high frequency vocalizations were still evident at P5 and P7. However, by age P9, the duration and frequency of vocalizations in CBA and CBADbh are virtually indistinguishable.

Comparison of vocalization frequencies in CBA, CBADbh+/-, and CBADbh+/+ mice

Since the CBADbh pup calls seem to be similar to CBA pup calls, quantitative tests on individual acoustic parameters were done to further test for similarity. In order to test the hypothesis that CBADbh+/- mice have acoustic features similar to the wild-type mice, CBADbh+/- and CBADbh+/+ were separately grouped and analyzed along with the CBA mice. Unexpectedly, there was a significant difference in the vocalization frequencies for CBA, CBADbh+/-, and CBADbh+/+ mice with a p-value of < 0.05 when analyzed with the Kruskal-Wallis test. Since the samples originated from a relatively small number of animals, with large amounts of sample points obtained from each animal, significant differences could be driven by individual animal variation in vocalization frequencies. Furthermore, the calls generally fell into two clusters of acoustic frequencies, a low and a high range of frequency. Clustering of these low or high range call frequencies were apparent in the probability call clouds of CBA and CBADbh

pups. To more accurately separate these clusters into groups of low or high frequencies, the vocalization frequency scatter plot was visually examined. 85 kHz was chosen as the threshold for high frequency USVs because it contains the least number of calls between the two clusters under visual inspection of the scatter plot and histogram which represents the number of calls at each frequency (Fig. 5a,b). To prevent oversimplification and misrepresentation of bimodal data of call frequencies, the necessity to distinguish between low and high frequency pup ultrasonic vocalizations is evident.

Basic acoustic parameters of CBADbh pup vocalizations

To address the animal variances in pup calls as seen in the previous frequency analysis, all further analyses were done on a per animal basis (call parameters averaged within animal), instead of per call basis as was done in the clouds to prevent skewed results. Separately for the low and high frequency USV groups, call parameters were averaged within animals then compared across animal groups. All statistical tests used in these analyses were the non-parametric Steel-Dwass method. Contrary to the significant result from previous analysis of pup calls, after averaging within animal, averaged high frequency USVs (Fig. 7a) were not statistically different ($P > 0.05$) between CBA, CBADbh+/- and CBADbh+/+ mice across all ages. Averaged low frequency USVs were also non-significant ($P > 0.05$) between all groups across all ages (Fig. 7b). This suggests that individual variability in animals' call frequency drove our initial vocalization frequency analysis to significance.

Call durations were similarly pooled and averaged within each animal for analysis (Fig. 7c). These results yielded no statistical significance between call durations of CBA, CBADbh+/-, and CBADbh+/+ mice. The lack of significance ($P > 0.05$) in call durations suggest the longer

durations seen in the CBADbh versus CBA call clouds were due to individual variation, and not due to group differences.

CBADbh pup vocalization rates during development

CBADbh+/- and CBADbh+/+ mice were compared to determine if there were any significant group differences in call rates due to the CBADbh+/- genotype. There was a general trend of increasing call rates as pups aged in the two groups, but call rates (Fig. 8a) of CBADbh+/- vs. CBADbh+/+ for each day were not significantly different ($P > 0.05$, Wilcoxon rank-sum). Although unable to determine statistical significance, the only CBADbh-/- pup in the study had a much lower vocalization rate when compared to the CBADbh+/- and CBADbh+/+ mice (Fig. 8b). As mouse pups increase vocalization rates with development (Hahn et al., 1998), this is in line with previous findings that Dbh-knockout mice are developmentally delayed (Thomas et al., 1995).

Comparison of ABR thresholds and proportion of hearing across strains

Due to the similarities in vocal properties between CBADbh+/- and CBADbh+/+, it could be argued that these two groups can be grouped into one NE-competent animal group. Taking into consideration the small group sizes, CBADbh NE-competent mice were pooled for ABR analyses for stronger statistical power. CBA and CBADbh NE-competent mice have better hearing than the Dbh+/- C57BL/6J and 129/SvEv mixed mouse strain (C57/129Dbh+/-), especially in the high frequency ranges. The proportion of hearing for the three groups, CBA, CBADbh NE-competent, and C57/129Dbh+/- were significantly different at higher frequencies (32 kHz, 64 kHz, 80 kHz) with $P < 0.05$ using the Pearson's Chi-Squared test (Table 1).

C57/129Dbh mice had a much lower percentage of hearing as compared to CBA and CBADbh mice. These results suggest the success of backcrossing as CBADbh NE-competent mice have hearing capabilities more similar to the CBA than the C57/129Dbh+/- mice. Success of backcrossing could be determined by hearing thresholds as well as hearing capability at each frequency.

Click hearing threshold for both CBA mouse strain and the CBADbh strain were significantly lower than the C57 strain when controlled for multiple group comparisons ($P < 0.05$, Steel-Dwass). Among animals that could hear at each frequency, hearing threshold of CBADbh mice was significantly poorer than the C57 mouse strain at 8 kHz stimuli with $P < 0.05$ using Steel-Dwass method (Fig. 9a). In evidence of backcrossing success, CBA and CBADbh hearing were not significantly different in all ABR comparisons ($P > 0.05$ in all cases, Steel-Dwass). Because of the differences in hearing proportions at high frequencies, there could be a potential bias in threshold comparisons. The failure to show group differences between C57/129Dbh+/- and CBADbh high frequency hearing thresholds (Fig. 9b) was attributed to a higher proportion of C57/129Dbh strain incapable of hearing higher frequencies (32 kHz, 64 kHz, 80 kHz).

CBADbh-/- hearing proportion and ABR thresholds as compared to CBADbh+/- and +/+ mice

In order to observe the effects of Dbh genetic knockout on audition, the only CBADbh-/- mouse available in this study was plotted on a summarized line graph of CBA, CBADbh (both +/- and +/+), and C57/129Dbh+/- group hearing proportions and thresholds (Fig. 10a,b). The CBADbh-/- mouse was capable of hearing at all stimulus frequencies except at 80 kHz. Hearing thresholds in response to click stimulus and pure tone stimuli between 8 kHz and 64 kHz were

comparable with CBA and CBADbh mice. This suggests that it is possible for a CBADbh-/- mouse to hear at ultrasonic frequencies comparable to the CBA background strain.

Discussion

The goal of this project was to establish the CBADbh model for studies on NE in the auditory cortex, to be used specifically in a social context. The mouse model was chosen because of its ease of genetic manipulations and gene backcrossing. Dopamine beta hydroxylase knockout mice were used to selectively lower NE levels in both the central nervous system and periphery. The Dbh KO mice that were previously used in our lab to study experience-dependent plasticity during development (between P7 and P21) were bred on a C57BL/6J and 129/SvEv background (Shepard et al., 2015a). These knockout mice had impaired map plasticity, but these results were found in an experimental paradigm that was not socially relevant to the mice. In order to study NE involvement in a social context, it was imperative that mice had high frequency hearing, and thus capable of hearing pup calls that are salient. The CBADbh mouse model was generated for a way to manipulate NE levels, and capable of naturalistic design paradigms in a social context. In establishing the model, CBADbh pup vocalizations were determined to be qualitatively similar to CBA pup vocalizations. Furthermore, analyses on CBADbh capability of hearing showed that CBADbh hearing resembled CBA mice, and were better than C57/129Dbh mice at high frequencies. The results of this study on vocalizations and hearing of the CBADbh mice supports the idea that it is a good replacement to the C57/129Dbh mice previously used in the lab for auditory cortical studies involving NE.

Call rates were not different between CBADbh+/- and CBADbh+/+ mice. However, it is interesting to note that the one CBADbh-/- mouse available in this study showed markedly lower call rates compared to its NE-competent CBADbh counterparts. The KO mouse did not vocalize at P5, and at P7 it vocalized at a rate of 0.09 calls/sec, an 86% decrease from the call rates of

CBADbh+/+ mice. Although it is unwise to draw conclusions from a single animal, the KO mouse was useful in giving trends we can expect to see in future studies on KO mice. The lower call rate of CBADbh-/- when isolated could be attributed to the general low arousal of NE-depleted mice (Foote et al., 1980). It should be noted that isolation-induced pup USVs are a known behavioral response to combat cold exposure, along with the physiological response of thermogenesis by brown adipose tissue (Smith, 1964, Allin and Banks, 1971). However, Dbh KO mice are unable to induce thermogenesis in brown adipose tissue due to impaired peripheral vasoconstriction, yet the CBADbh-/- mouse vocalized at a much lower rate despite being cold intolerant (Thomas and Palmiter, 1997b). To explain this paradox, developmental differences in Dbh KO mice must be considered. Since young rodents early in development can better withstand hypothermia and hypoxia, thermoregulatory responses such as USV production are not yet necessary for the survival of young rodent pups (Okon, 1970b, Okon, 1970a, Blumberg and Alberts, 1990). It is plausible then that the CBADbh KO mouse vocalized at a much lower rate because of its developmentally delayed phenotype. Future studies that follow and record individual KO pup USVs up until P15 would be interesting, as it would show the differences in vocalization rates as KO pups go through development, as compared to the NE-competent pups.

Although the call probability clouds of CBA and CBADbh mice at P5 and P7 were slightly different, by P9 the call clouds overlapped almost entirely. This variability in call frequencies at age P5 and P7 is in line with previous findings that pup calls during the early stages of development are more variable and seem to gain consistency around P9 (Liu et al., 2003). In our initial USV comparison between CBA, CBADbh+/- and CBADbh+/+ mice, there were significant group differences. One explanation for this would be the small number of animals in my study. Since the USV comparison was done on a per call basis, a large number of

calls by an individual animal may be over-represented, amplifying individual variability and possibly driving significance in this analysis. Considering the nice overlap between CBA and CBADbh call clouds (Fig. 6a), it was unexpected that there were significant group differences between CBA vs. CBADbh+/- and CBA vs. CBADbh+/+ pup USVs when each animal's call was considered individually. As expected, after averaging calls within animal into groups of low or high frequency calls, no significant differences are seen between CBA, CBADbh+/-, and CBADbh+/+ pups. Importantly, there were no significant differences between the two CBADbh genotypes, supporting the initial hypothesis that a single mutant Dbh allele in the CBADbh+/- mice does not affect normal USV production when compared to the CBADbh+/+ mice. To my knowledge, this is the first study that characterized the vocal properties of Dbh+/- vs. Dbh+/+ mice. This is consistent with previous reports that heterozygous Dbh+/- mice are phenotypically the same as wild-type Dbh+/+ (Thomas et al., 1998, Bourdelat-Parks et al., 2005).

ABR results on hearing capabilities of mice strains were supportive of backcrossing success. CBADbh NE-competent mice, unlike the previous C57/129Dbh mice, were more capable of hearing at high frequencies, to levels comparable with the background CBA mouse strain. Although the analyses on ABR measurements of high frequency hearing thresholds were non-significant, this can be attributed to the low percentage of mice capable of high frequency hearing in the C57BL/6J and 129/SvEv mixed background strain. To circumvent this problem in the future, speaker output could be raised higher for all mice groups at high frequencies. In doing so, we might find numerical values of hearing thresholds for the previously non-hearing mice, which might better represent hearing threshold of mice instead of categorical data showing hearing or incapable of hearing. For this study, mice incapable of hearing a specific stimulus did not have a recorded numerical hearing threshold, and in order to include these animals,

categorical analysis of “Hearing” vs. “Non-hearing” needs to be conducted. Disappointingly, previous studies that compare hearing thresholds between strains did not consider the proportion of animals capable of hearing and instead only use hearing threshold, which out of necessity will exclude animals that are unable to hear. Information on how likely animal strains are able to hear (to any capacity) at a given frequency should be a supplement to hearing threshold analyses because it gives a clear and overall view of hearing capabilities of a strain.

Regarding the limitations of this study and the Dbh mutant strain itself, despite offering a cleaner way of depleting NE, there are minor problems with genetic knockout of Dbh. Because the conversion of dopamine (DA) to NE is the main catabolic pathway of DA, the lack of the Dbh enzyme responsible for this conversion might lead to increased DA levels in Dbh-/- mice. As a consequence of lacking Dbh enzyme, DA levels are significantly increased in the central nervous system of Dbh-/- mice (Bourdelat-Parks et al., 2005). The increase in DA levels could potentially be circumvented by administering DA antagonists. However, this will not be included in the near future regarding experiments on CBADbh-/- mice, as it is unclear whether abnormal DA levels could significantly affect the results we are interested in. Another limitation of this study is the small number of animals available. Perhaps with a larger sample size, the significant differences would cease to appear in the USV analyses as individual differences will be less amplified with larger numbers. On the contrary, if there was an actual difference, a larger sample size would show better trends of significance that are more convincing. More CBADbh KO animals will be needed to study mutant Dbh effects on USV production and hearing thresholds as well. The greatest limitation of this study was the failure to obtain a complete auditory cortical tonotopic map. A major challenge in complete cortical mapping is that it requires roughly 12 hours of continuous anesthesia. The ketamine/xylazine mix used for anesthesia lasts only about

an hour after the initial dose, and titrating anesthesia level by administering supplemental maintenance doses IP for the remainder of the procedure is challenging. Instead of ketamine/xylazine, inhalation anesthetics such as isoflurane are typically used to titrate anesthesia levels for longer procedures. However, in auditory cortical mapping, isoflurane is typically avoided as studies had shown isoflurane-induced anesthesia to reduce cortical sensitivity (Madler et al., 1991, Cederholm et al., 2012). Because we were unable to obtain a tonotopic map, it is still unclear whether CBADbh mice exhibit auditory cortical tonotopy similar to the CBA mice.

Successful backcrossing yielding CBADbh mice comparable with CBA mice in hearing and USV production has great implications. We can directly study the effects of NE depletion on USV production by using the genetic knockout CBADbh-/- mice, which would give strong evidence into the role of NE in vocal production. Furthermore, the greater ability to hear at higher frequencies makes the CBADbh mice an attractive model to study NE involvement in mouse social vocal communication. An example of such would be the maternal responses to pup USVs. As previously discussed in the introduction, pup USVs are between 30 kHz and 90 kHz, which are highly salient cues that will trigger maternal pup retrieval response in caring mothers (Ehret and Haack, 1981, Ehret, 2005). We can use the natural pup-mother vocal communication paradigm to study the perception of pup USVs in mothers.

One possibility is that we can potentially combine CBA nursing cages (mothers with their pups) and adult female CBADbh-/- naïve mice to study the KO mice's cocaring ability. Cocarers are naïve mice (mice with no prior experience with pups), that are placed with the mother in the nursing cage, which are useful for studying experience-dependent plasticity independent of the

maternal physiological state (Lin et al., 2013). It is important to note that although cocarers do not undergo physiological changes as in motherhood, prolonged pup exposure is sufficient to enhance neural processing in response to pup calls (Miranda et al., 2014). The capability of CBADbh KO mice as cocarers raise an interesting question because Dbh KO mothers were known to have deficits in maternal behavior (Thomas and Palmiter, 1997a). However, Dbh KO mice were able to overcome its maternal deficits only if NE was present at the time of parturition, suggesting NE's role in learning emotional salience. A cocarer study would be able to answer whether the same underlying neural mechanism causing KO maternal deficits also prevent experience-dependent learning of pup call salience in naïve Dbh KO mice. This future study would have a significant impact as it will shed some light into NE involvement in adulthood experience-dependent plasticity of the subcortical auditory pathway in a social environment. Auditory cortical tonotopic maps will need to be obtained to determine whether CBADbh mice are similar on the auditory cortical map level as CBA mice.

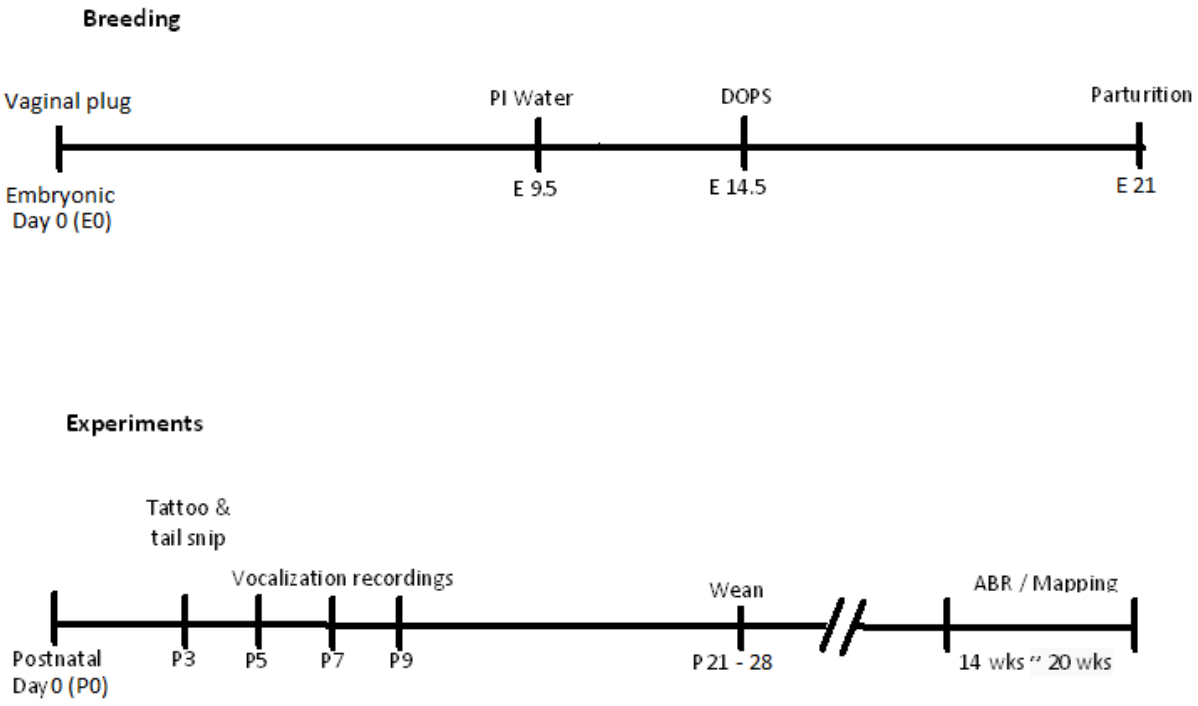

Figure 1. Timeline of breeding and experimental procedures.

Phenylephrine + Isoproterenol (PI water) was given in drinking water at embryonic day 9.5 (E9.5) and L-threo-3,4-dihydroxypheylserine (DOPS) was given at E14.5. For experiments, tattoo and tail snip was done at P3. Vocalizations were recorded at P5, P7, and P9. Animals were weaned at P21 or P28 if CBADbh knockouts were present. Auditory brainstem response (ABR) was recorded on the offspring between 14-20 weeks after birth. Auditory cortical tonotopic mapping was done after ABR.

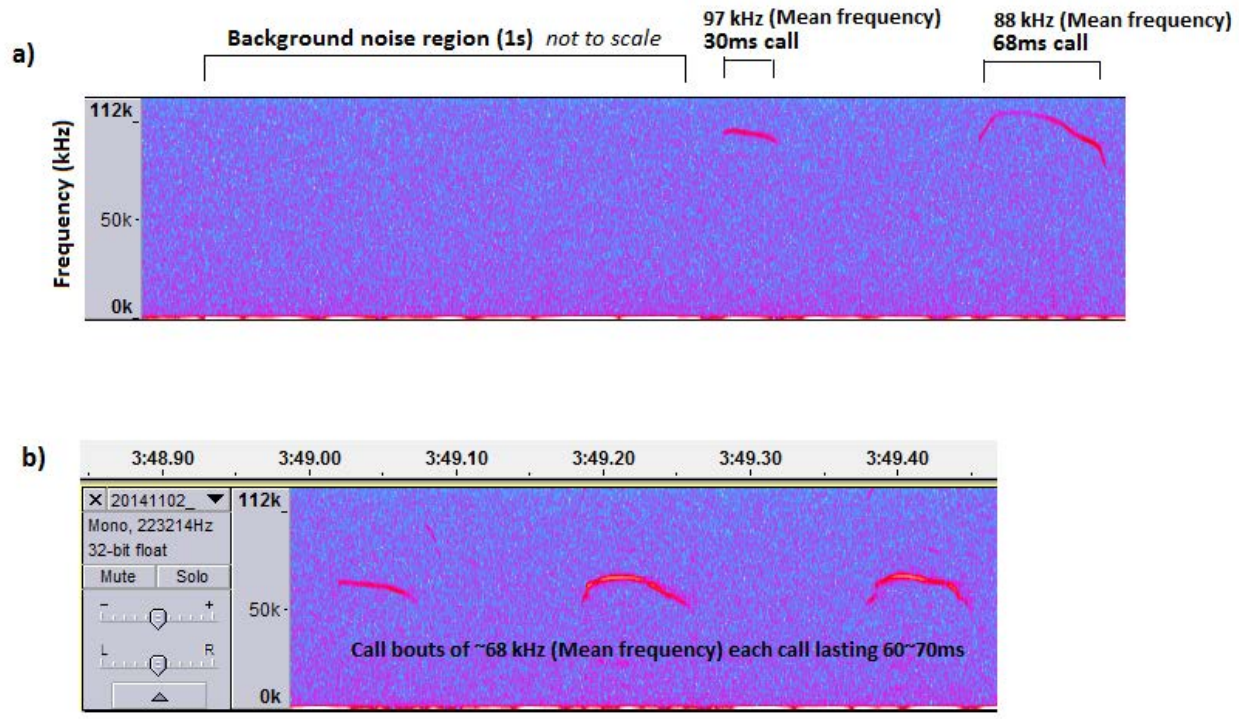

Figure 2. Sample spectrograms of pup ultrasonic vocalizations

a) Sample spectrogram with an example of a background noise region of about 1 second (not to scale). Examples of high frequency vocalizations are annotated with its mean frequency (kHz) and duration (ms). **b)** Sample spectrogram that is to scale; Call bouts of low frequency ultrasonic vocalizations (<85 kHz) are shown, with each call lasting about 60~70ms and a mean frequency of ~68 kHz.

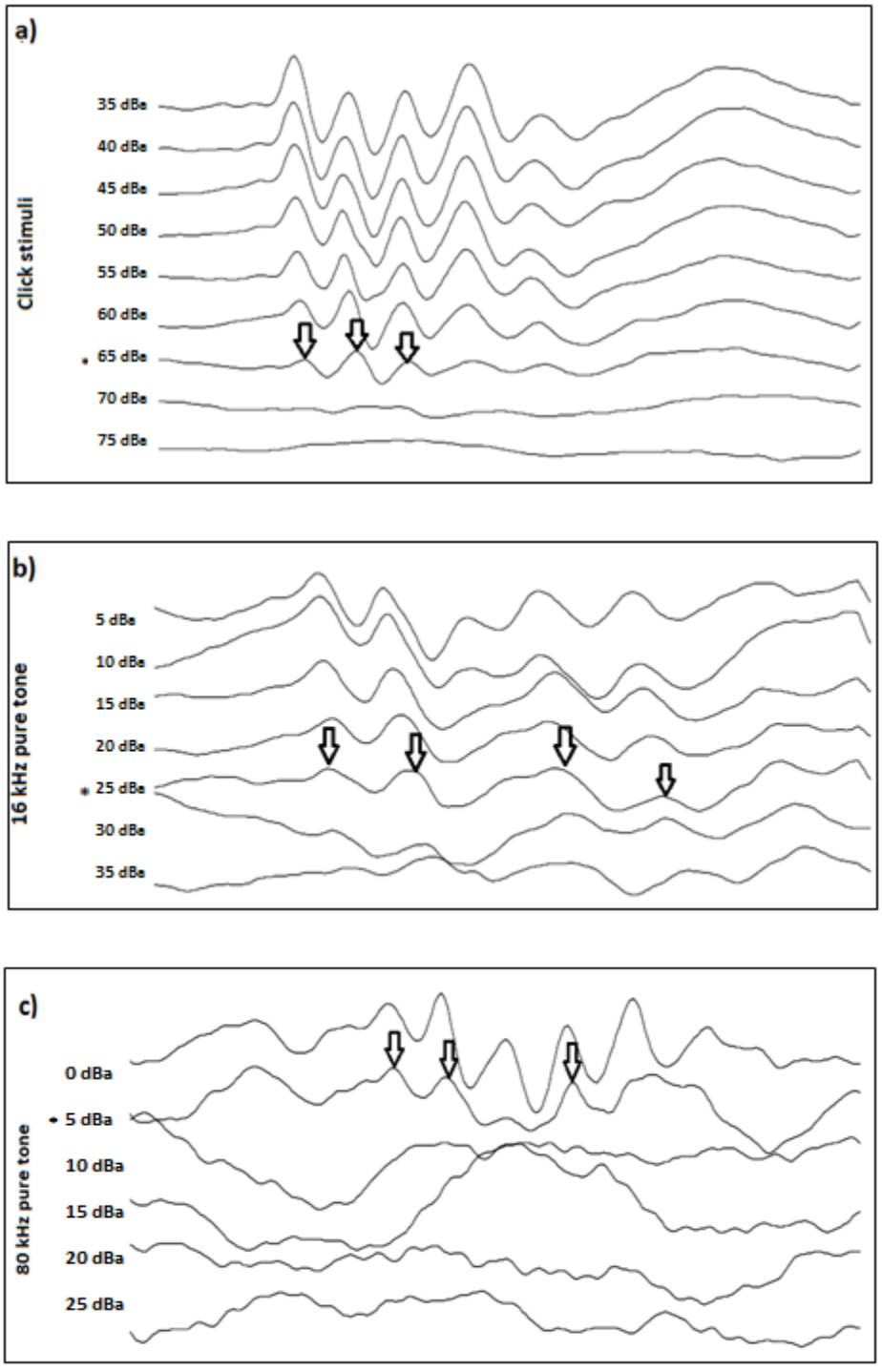

Figure 3. Sample ABR thresholds

a) Hearing threshold for click stimuli was determined to be at 65dBa (* denotes hearing threshold). **b)** Hearing threshold for 16 kHz pure tone was determined to be 25dBa. **c)** Hearing threshold for 80 kHz pure tone was determined to be 5dBa.

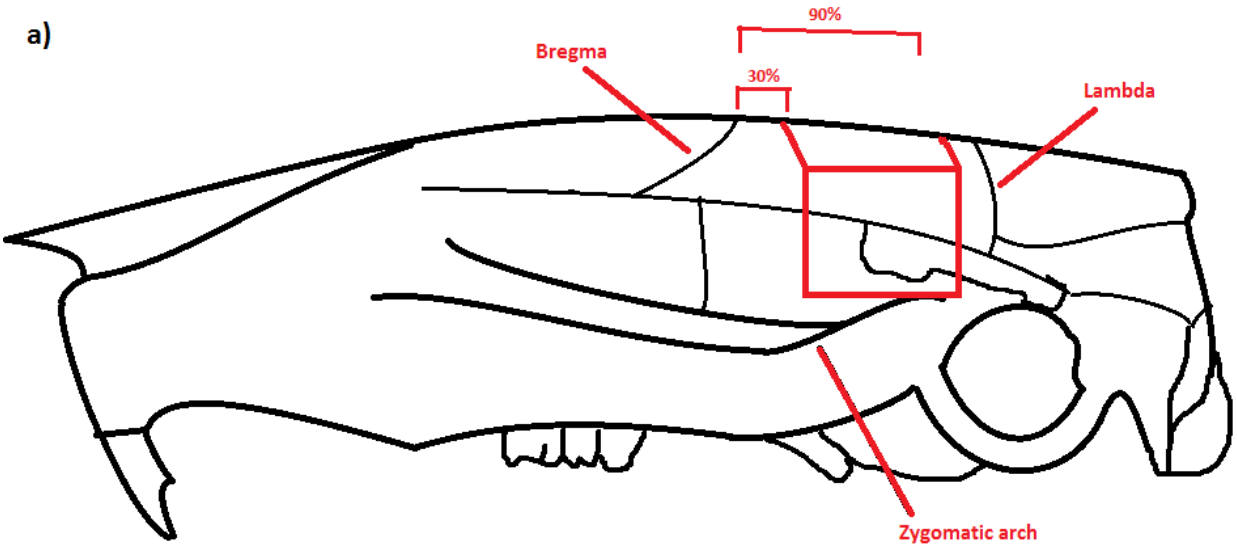

Figure 4. Targeted area for craniotomy

(a) Targeted cranial window is boxed red in the figure depicting mouse skull anatomy (Image adapted from http://www.informatics.jax.org/cookbook/figures/figure12.shtml). Lambda and bregma (In red) are used as landmarks to consistently target the cranial window over the auditory cortex. **(b)** Sample craniotomy revealing auditory cortex

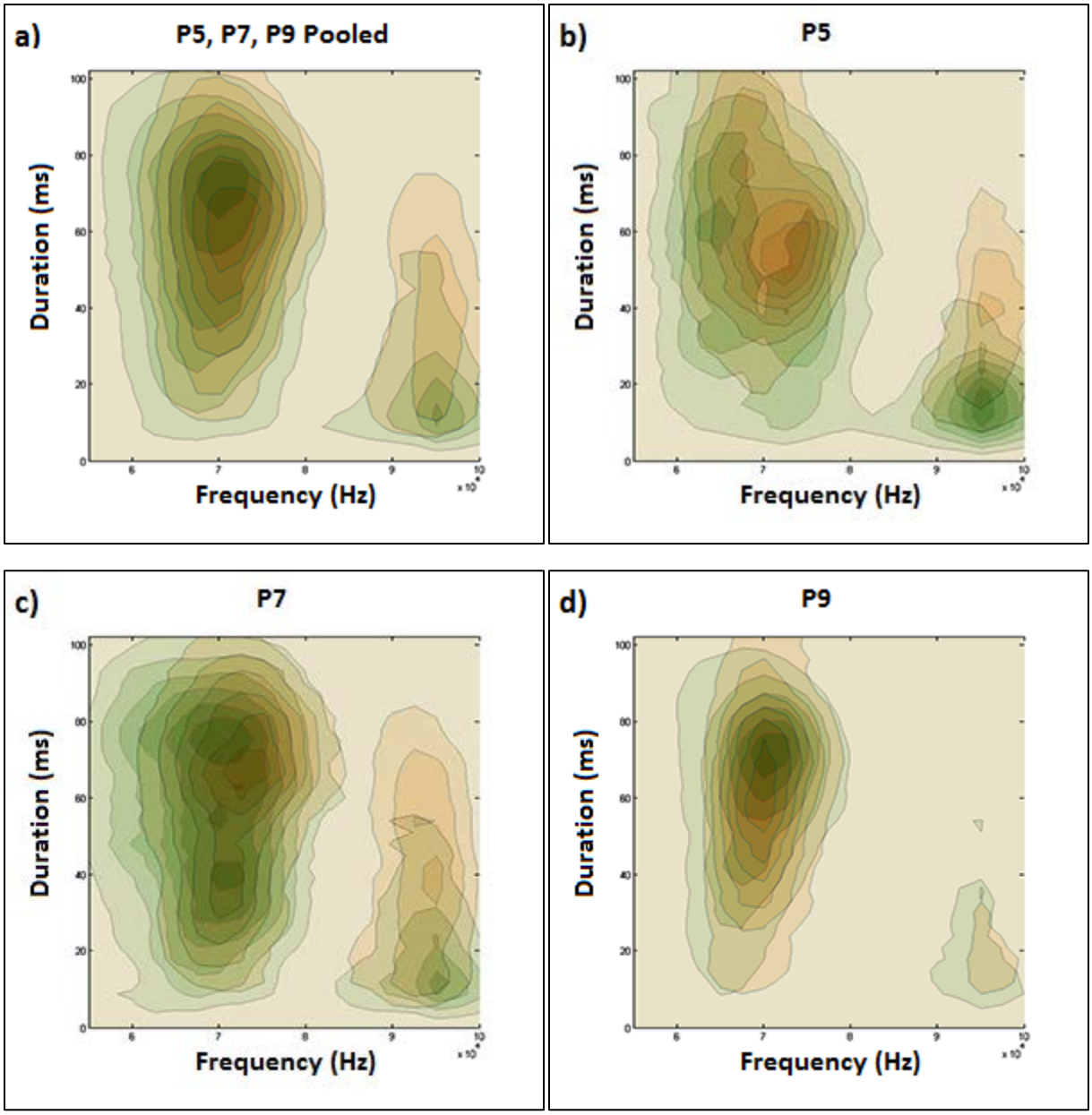

Figure 5. Comparison of CBADbh and CBA pup vocalizations

(a) Pooled probability call clouds of CBADbh (+/- and +/+), labeled orange; CBA calls labeled green. **(b)** P5 probability call clouds (**c)** P7 probability call clouds (**d)** P9 probability call clouds.

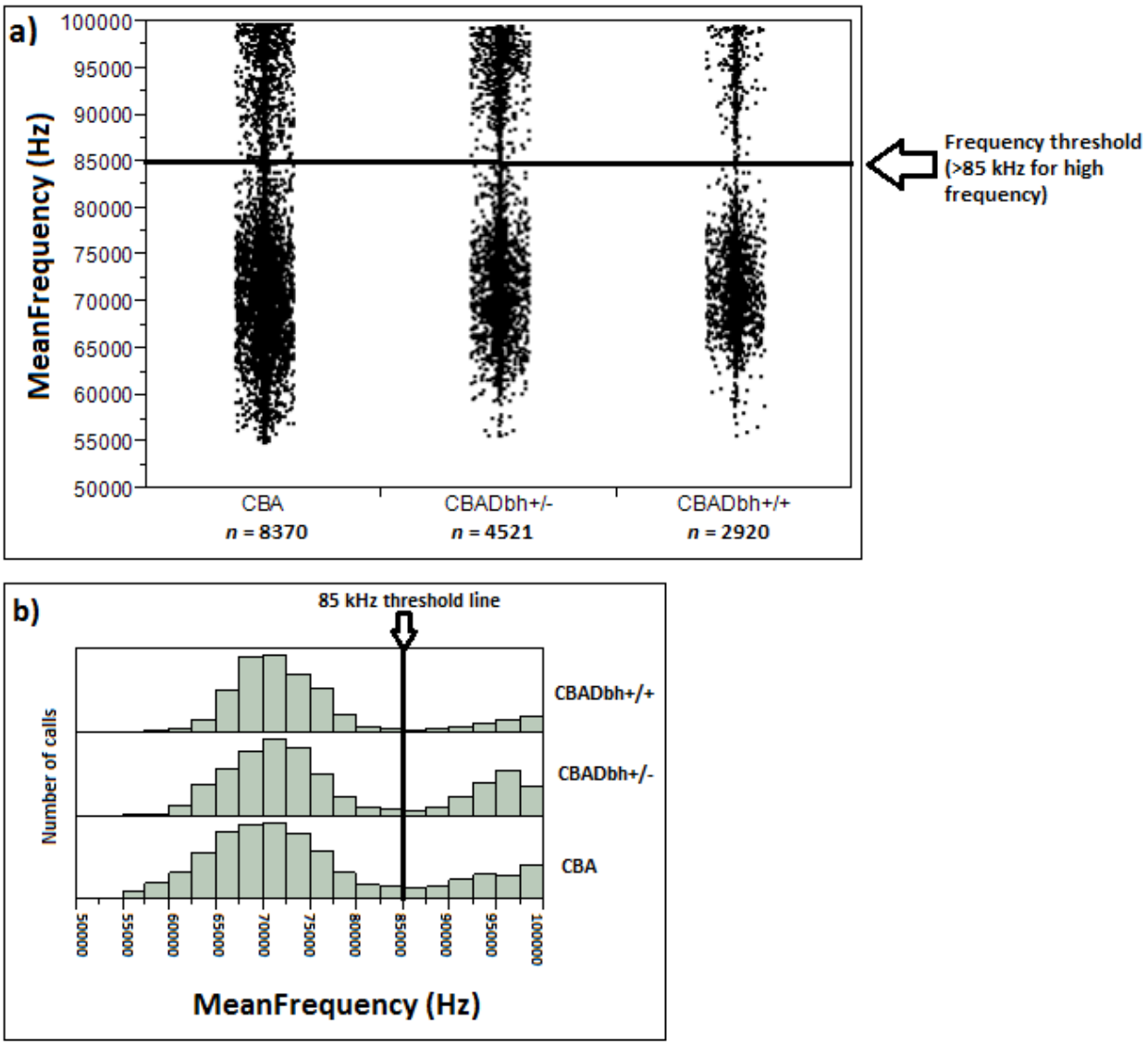

Figure 6. Mean frequency of CBA and CBADbh pup vocalizations

(a) Scatter plot of the frequency of all ultrasonic vocalizations for CBA (*n* = 4697), CBADbh+/- (*n* = 4521), and CBADbh+/+ mice (*n* = 2920). There are significant group differences in mean frequency (***P < 0.05 for all groups**, Kruskal-Wallis). Line depicted is the frequency threshold for high frequency vocalizations as determined through visual inspection. **(b)** Histogram showing the least number of calls at 85 kHz, as depicted by the 85 kHz threshold line.

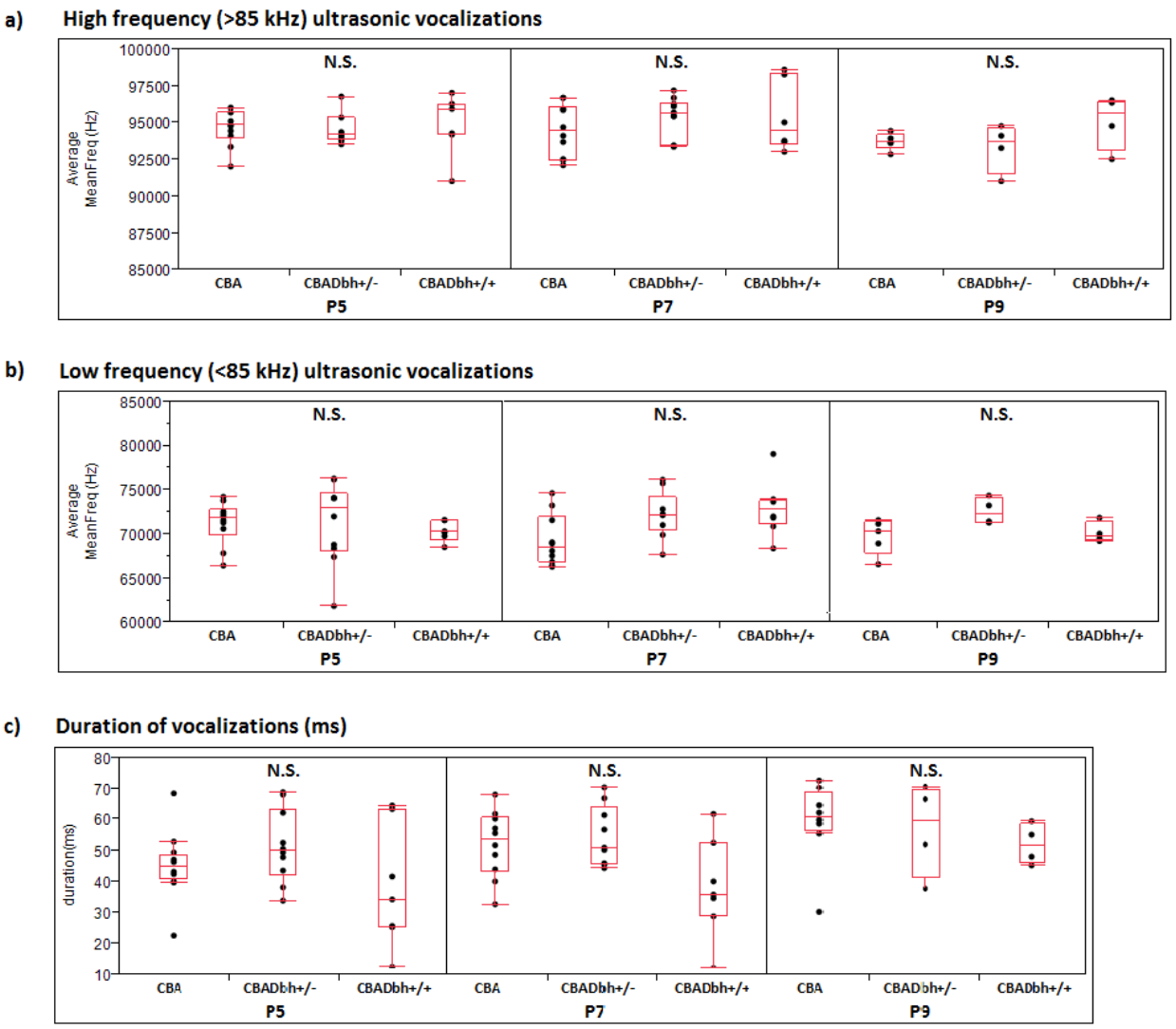

Figure 7. Basic acoustic parameters of CBA and CBADbh pup vocalizations

(a) Mean high frequency (>85kHz) ultrasonic vocalizations of individual CBA, CBADbh+/- (*het)* and CBADbh+/+ (*wt)* pups across P5 (CBA *n* = 10; *het n* = 10; *wt n* = 6), P7 (CBA *n* = 10; *het n* = 9; *wt n* = 7), and P9 (CBA *n* = 5; *het n* = 4; *wt n* = 4) over a period of 5 minutes (**N.S.** denotes non-significant P-value of > 0.05 for all groups; Steel-Dwass). **(b)** Mean low frequency (<85kHz) ultrasonic vocalizations of individual CBA, *het* and *wt* pups across P5 (CBA *n* = 10; *het n* = 10; *wt n* = 7), P7 (CBA *n* = 10; *het n* = 9; *wt n* = 7), and P9 (CBA *n* = 5; *het n* = 4; *wt n* = 4) over a period of 5 minutes. **(c)** Mean call duration of of individual CBA, *het* and *wt* pups across P5 (CBA *n* = 12; *het n* = 10; *wt n* = 7), P7 (CBA *n* = 10; *het n* = 9; *wt n* = 7), and P9 (CBA *n* = 8; *het n* = 4; *wt n* = 4).

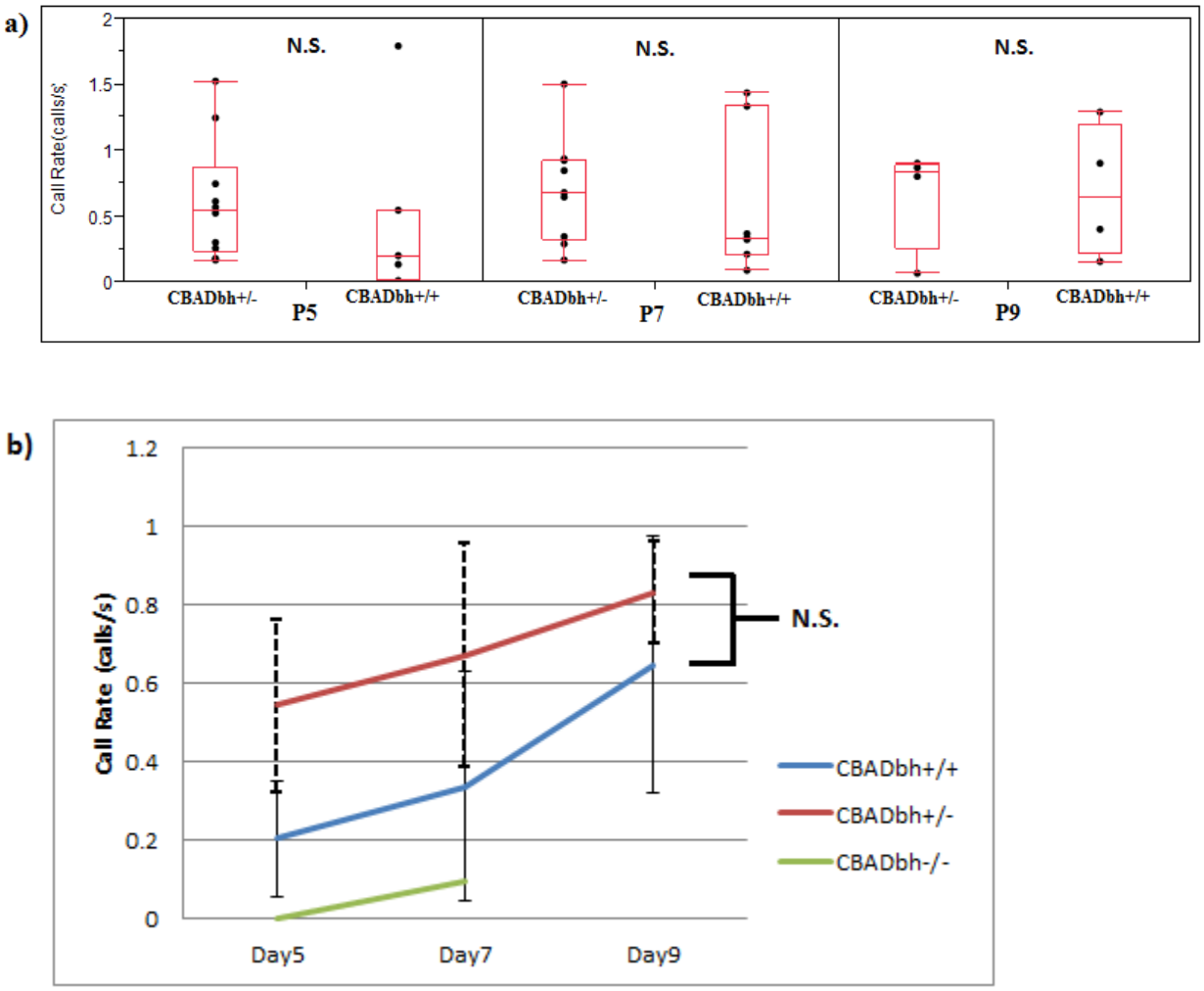

Figure 8. CBADbh pup vocalization rates

(a) Similarities in call rates (number of vocalizations per second) in individual CBADbh+/- (*het)* and CBADbh+/+ (*wt)* pups across P5 (*het n* = 10; *wt n* = 7), P7 (*het n* = 9; *wt n* = 7), and P9 (*het n* = 4; *wt n* = 4); (P > 0.05 in all cases, Steel-Dwass). **(b)** Averaged call rates for each genotype were plotted across the 3 days to visualize the general trend of call rates, and to show the call rate of an individual CBADbh-/- (*n* = 1) pup as compared to the average call rates of CBADbh+/- and CBADbh+/+ mice from figure (a). Differences between CBADbh+/- vs. CBADbh+/+ were not significant (P > 0.05, Wilcoxon rank-sum test).

Stimulus / Group	Click	8kHz	16kHz	* 32kHz	* 64kHz	* 80kHz
% ResponsiveC57+/- (n=8)	100%	100%	88%	50%	63%	50%
% ResponsiveC57-/- (n=8)	75%	75%	75%	13%	13%	13%
% ResponsiveCBA (n=5)	100%	100%	80%	100%	80%	75%
% ResponsiveCBADbh (n=9)	100%	100%	100%	100%	89%	67%
% ResponsiveCBADbh-/- (n=1)	100%	100%	100%	100%	100%	0%

Table 1. Percentage of hearing response. Groups had significantly different proportionate hearing at higher frequencies (32kHz, 64kHz, and 80kHz; ***P < 0.05**, Pearson's chi-squared). Green cells are hearing percentages of 75% - 100%, yellow cells are between percentages of 50% to 75%, and red cells denote poor likelihood of hearing at 0% to 49%.

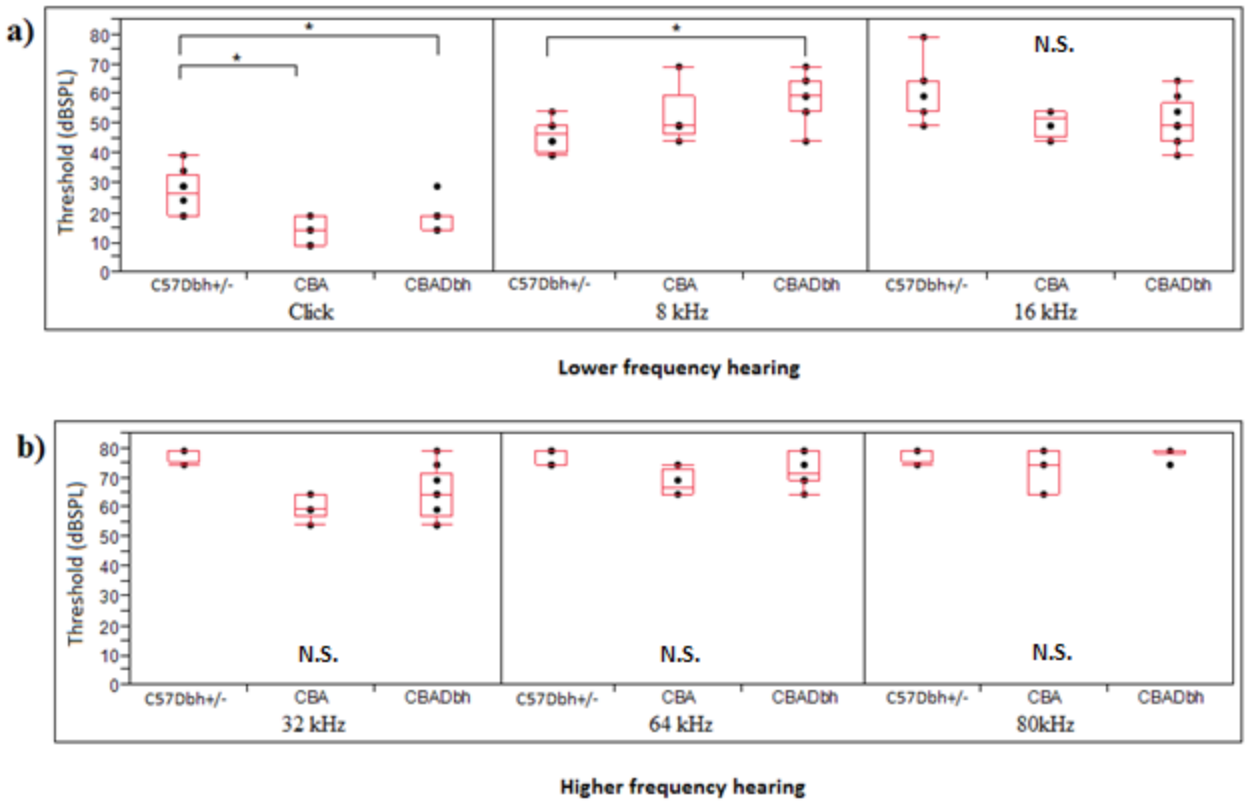

Figure 9. ABR threshold comparisons between C57/129Dbh+/- vs. CBA vs. CBADbh mice

(a) Hearing threshold of click and lower frequency stimuli for C57+/- (*n* = 8), CBA (*n* = 5), and CBADbh (+/+ and +/-; *n* = 9). CBA and CBADbh had significantly better hearing for click stimuli than C57/129Dbh+/- mice (***P < 0.05**; Steel-Dwass). CBADbh had significantly poorer hearing than C57/129Dbh+/- mice at 8kHz (***P < 0.05**; Steel-Dwass)

(b) Hearing threshold of higher frequency stimuli for C57+/- (*n* = 8), CBA (*n* = 5), and CBADbh mice (+/+ and +/-; *n* = 9). No significant differences in all groups (P>0.05; Steel-Dwass).

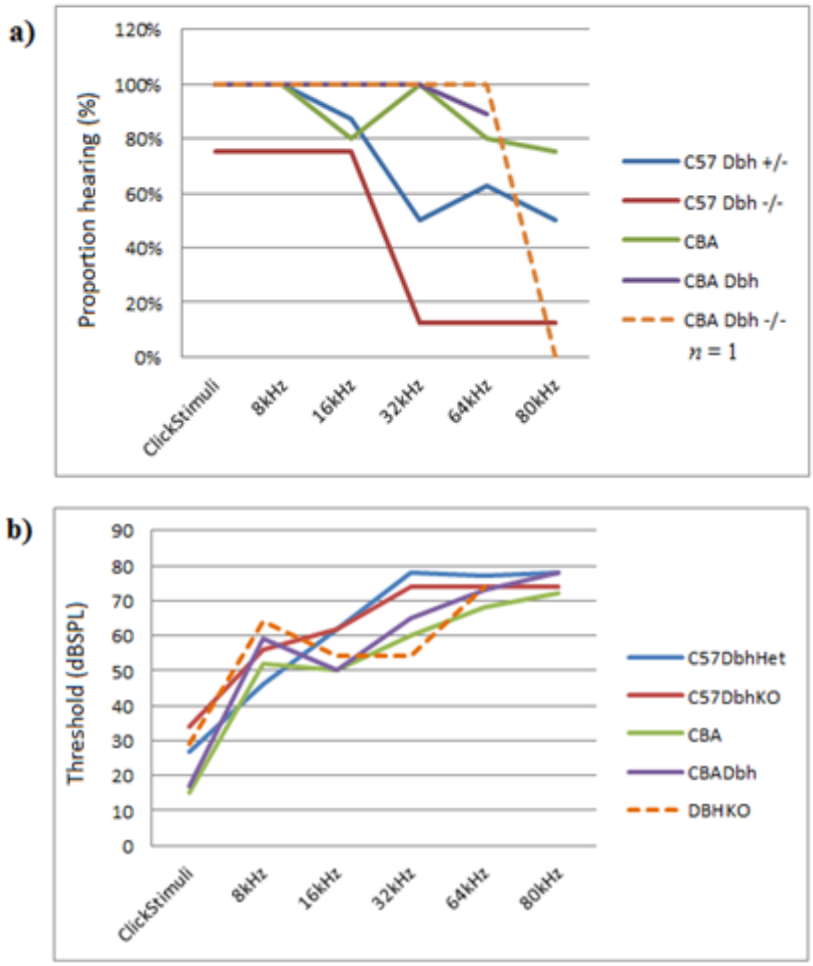

Figure 10. Comparison of ABR thresholds and proportion of hearing across strains

(a) Proportion of mice able to hear during ABR stimuli. (C57/129Dbh+/- *n* = 8; C57/129Dbh-/- *n* = 8; CBA *n* = 5; CBADbh+/+ *n* = 5; CBADbh+/- *n* = 4) Hearing ability of one CBADbh-/- mouse was plotted as a dotted line.
(b) Average hearing thresholds of mice that were responsive to stimuli. Note the CBADbh-/- mouse was not able to hear at 80 kHz.

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