

Research paper

# Auditory brainstem responses are impaired in EphA4 and ephrin-B2 deficient mice

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## Abstract

The Eph receptor tyrosine kinases and their membrane-anchored ligands, ephrins, are signaling proteins that act as axon guidance molecules during chick auditory brainstem development. We recently showed that Eph proteins also affect patterns of neural activation in the mammalian brainstem. However, functional deficits in the brainstems of mutant mice have not been assessed physiologically. The present study characterizes neural activation in Eph protein deficient mice in the auditory brainstem response (ABR). We recorded the ABR of EphA4 and ephrin-B2 mutant mice, aged postnatal day 18–20, and compared them to wild type controls. The peripheral hearing threshold of EphA4<sup>-/-</sup> mice was 75% higher than that of controls. Waveform amplitudes of peak 1 (P1) were 54% lower in EphA4<sup>-/-</sup> mice than in controls. The peripheral hearing thresholds in ephrin-B2<sup>lacZ/+</sup> mice were also elevated, with a mean value 20% higher than that of controls. These ephrin-B2<sup>lacZ/+</sup> mice showed a 38% smaller P1 amplitude. Significant differences in latency to waveform peaks were also observed. These elevated thresholds and reduced peak amplitudes provide evidence for hearing deficits in both of these mutant mouse lines, and further emphasize an important role for Eph family proteins in the formation of functional auditory circuitry.

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**Keywords:** Brainstem; ABR; Eph receptor; Ephrin; Mouse

## 1. Introduction

Eph receptor tyrosine kinases and their membrane-anchored ligands, ephrins, constitute a large family of molecules that mediate intercellular signaling, with broad functions during development. In the nervous system, these proteins regulate cell migration (Krull et al., 1997; Mellitzer et al., 2000), axon outgrowth (Kullander and Klein, 2002; Murai and Pasquale, 2003; Wilkinson, 2001), synapse formation and stability (Dalva et al., 2007), and synaptic plasticity (Contractor et al., 2002; Dalva et al., 2007; Henderson et al., 2001). These functions for Eph proteins contribute to the orderly patterning and connectivity seen in the auditory system. In the ear, Eph proteins act as axon guidance molecules in developing spiral ganglion neurons

(Bianchi and Gray, 2002; Brors et al., 2003), and mutations in EphB receptors result in altered distortion product otoacoustic emissions (Howard et al., 2003). Eph proteins are expressed in central auditory structures and have a role in auditory brainstem development (Cramer, 2005). Misexpression of Eph receptors in chick embryos results in targeting errors in the chick brainstem (Cramer et al., 2006, 2004; Huffman and Cramer, 2007). Moreover, studies of mice with mutations in Eph genes show that these molecules regulate patterning in the mammalian brainstem after deafferentation (Hsieh et al., 2007).

Studies of Eph/ephrin protein functions must take into account the complexity of binding interactions and signaling mechanisms, which occur bidirectionally into both the Eph-expressing and ephrin-expressing cells upon contact. Eph receptors and ephrins are subdivided into A and B subclasses (Eph Nomenclature Committee, 1997). In general, ephrin-A ligands (1–6 in vertebrates) bind EphA

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receptors (1–10) and ephrin-B ligands (1–3) bind EphB receptors (1–6). The two exceptions to this rule are that EphA4 binds to ephrin-A ligands as well as ephrin-B2 and ephrin-B3 (Gale et al., 1996), and EphB2 binds to ephrin-B ligands and ephrin-A5 (Himanen et al., 2004). Binding between ephrins and Eph receptors occurs with high affinity and may mediate either attraction or repulsion (Pasquale, 2005). Because the Eph family is large and shows promiscuous binding between ligands and receptors, the effects of mutations in a single Eph gene are often subtle. An important question is whether mutations that cause anatomical abnormalities also result in a corresponding alteration of auditory function. Most of the studies on Eph proteins in the auditory system have focused on the auditory nerve and brainstem. We recently found that mice with mutations in *EphA4* or in *ephrin-B2* show significantly altered levels and patterns of activation in the auditory brainstem following pure tone stimulation (Miko et al., in press). In order to evaluate whether these mutations have a significant effect on hearing function, we performed auditory brainstem response (ABR) measurements on mice with mutations in *EphA4* or *ephrin-B2*, and compared these measurements to wild type littermate controls. We found that both mutations result in elevated ABR thresholds, and that latencies of some peaks were significantly altered. These results suggest that *EphA4* and *ephrin-B2* are required for normal hearing.

## 2. Materials and methods

### 2.1. Animals

The mice used in this study were deficient in either *EphA4*, a receptor tyrosine kinase, or *ephrin-B2*, a transmembrane ligand for *EphA4*. We used two strains of mice, which had mutations in either *ephrin-B2* or *EphA4*, and mutations were linked to  $\beta$ -galactosidase expression. The *ephrin-B2* mutant mice (Dravis et al., 2004) were bred in our colony and maintained on a CD-1/129 background. In this strain, the mutant allele encodes a membrane-bound ephrin-B2- $\beta$ -galactosidase fusion protein in which the cytoplasmic domain of ephrin-B2 has been deleted. Within this strain, ABR measurements were performed on *ephrin-B2*<sup>+/-</sup> or *ephrin-B2*<sup>lacZ/+</sup> mice, as the *ephrin-B2*<sup>lacZ/lacZ</sup> mice are not viable postnatally. To study the effects of mutations in *EphA4*, we used *EphA4* gene trap mice (Leighton et al., 2001) provided to us by Marc Tessier-Lavigne. These animals were maintained in our colony on a C57/Bl6 background. The mutant allele in this strain has a null mutation in *EphA4* and expresses cytoplasmic  $\beta$ -galactosidase, which is inserted downstream of the *EphA4* promoter region. ABR measurements were performed on wild type (*EphA4*<sup>+/+</sup>), heterozygous (*EphA4*<sup>+/-</sup>), and homozygous (*EphA4*<sup>-/-</sup>) mice. Mice were postnatal day (P) 18–20 at the time ABR recordings were made. At this age, mice are mature enough to produce an ABR waveform that includes all peaks present in the adult waveform (Song

et al., 2006), yet young enough to minimize the impact of age-related hearing loss, which can occur quite early in the C57/Bl6 strain (Parham, 1997). All procedures were approved by the University of California Animal Care and Use Committee (IACUC).

### 2.2. ABR recordings

Prior to experiments, tympanic sound pressure level (SPL, expressed in dB re 20 Pa) was calibrated from 100 Hz to 30 kHz in 100-Hz steps under computer control using a 0.5-in condenser microphone model #4134 (Bruel & Kjaer). In addition, we ascertained that the mice were responsive to acoustic startle stimuli. Mice were anaesthetized with ketamine (75–85 mg/kg) and xylazine (0.1–0.5 mg/kg) until insensitive to toe pinch, and placed in a double-walled sound-attenuating chamber (Industrial Acoustics Corp.). Recordings were acquired as differentially recorded scalp potentials. Subcutaneous steel-tipped electrodes were placed at the vertex (positive), and the parallel to the mastoid (negative) ipsilateral to the sound presentation. A ground electrode was attached to the tail. At the left ear, a speaker was placed inside the pinna, against the entrance to the ear canal. Stimulus delivery was controlled from outside the acoustic chamber, and clicks were generated by a MALab system (Kaiser Instruments, Irvine, CA) that prompted a 100  $\mu$ s square wave pulse ten times/second for each decibel level. The click stimulus was delivered in 5 dB steps from 20 to 60 dB, and thereafter in 10 dB steps until 100 dB. ABR signals were band pass filtered below 10 Hz and above 1000 Hz with a Grass P511K amplifier, acquired with MALab at a sampling rate of 1/0.02 ms, averaged online over 500 trials for each dB, and monitored in a 10 ms display window. The filtered signals were also sent to a digital oscilloscope that was triggered to the clicks, and where EKG activity was monitored as an indicator of health during recordings.

### 2.3. Data analysis

Each ABR averaged trace was saved as a vector number string containing 500 units, representing 10 ms of time for each dB SPL. For each mouse, all vectors representing the dB SPL stimulus set were exported to Excel for analysis. The first 2 ms portion of each trace was the pre-stimulus period. All traces were set to the baseline by obtaining the average voltage of the pre-stimulus period from each trace, and subtracting this average from each point in the stimulus-evoked voltage in the remaining 8 ms. This correction also reduced the influence of background noise and DC offset on the recorded trace. Threshold was defined as the first dB SPL in which peak structures emerged above baseline. These peaks were identified by their temporal correlation to peaks at the higher sound levels, where the waves are typically much larger in amplitude. Cases were excluded from threshold analysis if the background noise was large enough to mask the recognition of waves at

low sound levels. For measurements of wave amplitude and latency, the 100 dB trace was plotted individually and values were read directly from this plot. For measurements of amplitude, the wave peak was defined as the first value of maximum voltage within a wave. Peak amplitude was then measured as the difference between the baseline value at the bottom of the positive-going wave, and the maximum value in the peak (Walsh et al., 1986). Latency was defined relative to the onset of the stimulus, and peak latency was the time coordinate of the maximum wave amplitude value. All measurements were made blind to genotype. These threshold, amplitude and latency values

were then analyzed in JMP v4.0 for statistical significance. Data plots and figures were composed with JMP v4.0, Freehand v11.0, Adobe Illustrator v11.0, and Igor Pro v4.08.

#### 2.4. PCR and genotyping

We used PCR to determine the genotype of the mice used in this study. Tail clips were obtained after termination of the ABR recording. The procedures used are those reported previously (Dravis et al., 2004; Hsieh et al., 2007). Briefly, tail samples were digested overnight and DNA was

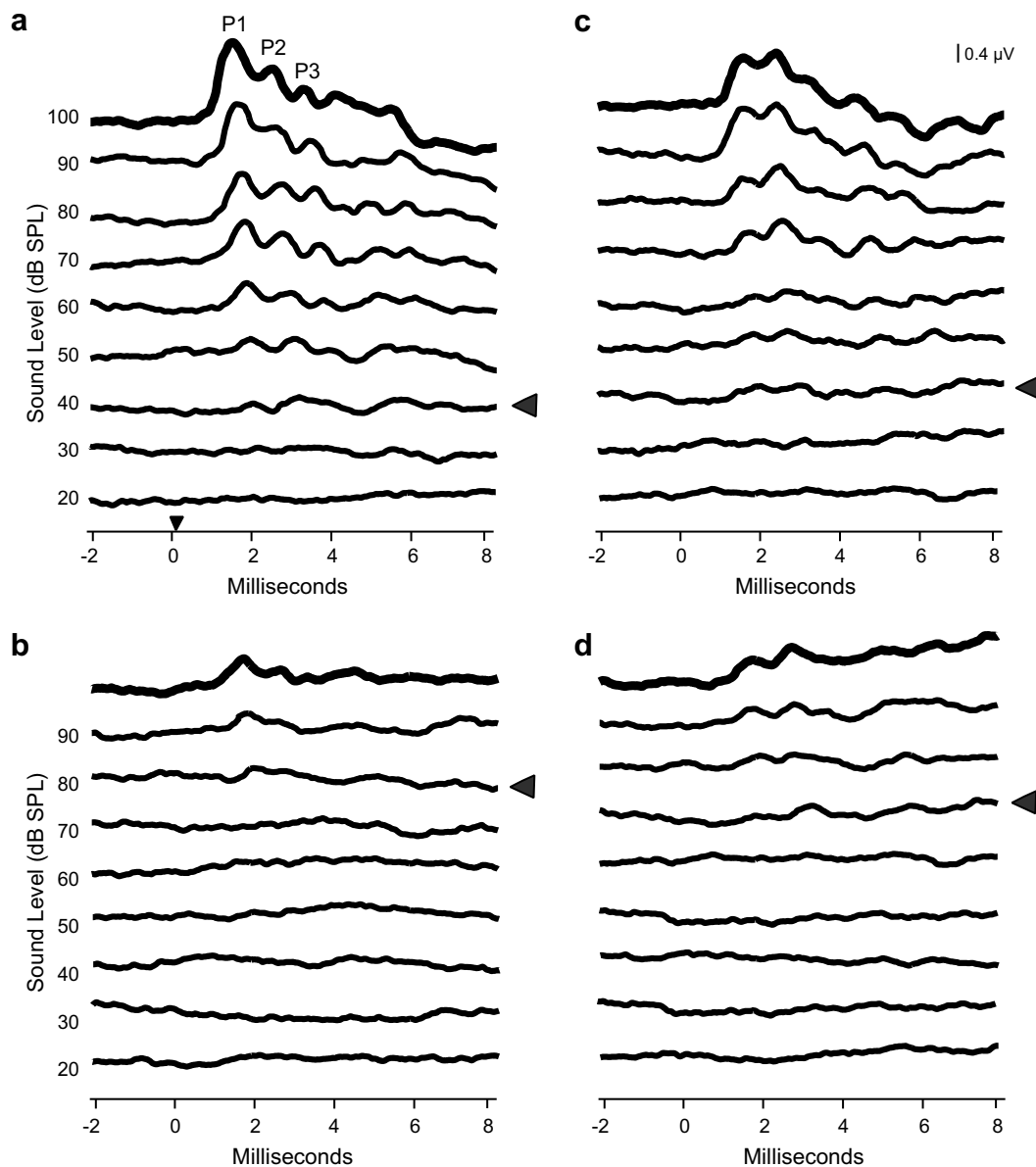


Fig. 1. ABR threshold. Averaged traces from each level of click presentation are shown in the same voltage scale, stacked in increasing dB SPL order. Large black arrowheads indicate ABR threshold. (a) A representative sample of traces from an *EphA4*<sup>+/+</sup> mouse shows an ABR with threshold near 40 dB. The small black arrowhead on the time axis indicates the approximate arrival of sound at the tympanic membrane (applies to all panels). (b) The *EphA4*<sup>-/-</sup> mouse shows a higher threshold, in this case close to 80 dB. In addition, the peak amplitudes appear smaller in the *EphA4*<sup>-/-</sup> (compare 100 dB traces in (a) and (b)). (c) Representative trace from an *ephrin-B2*<sup>+/+</sup> mouse. (d) Similar to the *EphA4* mutant, both an elevated threshold and reduced peak amplitude is seen in *ephrin-B2*<sup>lacZ</sup><sup>+/+</sup> mice as compared to wild type (compare 100 dB traces in (c) and (d)).

extracted with 100% isopropanol and resuspended in distilled water at a concentration of 0.3 mg/ml. The primers for EphA4 genotyping were: GTTTCCGCTCTGAGCT-TATACTGC, ACAGTGAGTGGACAAAGAGACAGG, and CGCTCTTACCAAAGGGCAAAC. The PCR amplification yielded a 639 bp band for the wild type allele and an 800 bp band for mutant allele. The primers for the ephrin-B2 mice were: AGGCGATTAAGTTGGG-TAACG, TCTGTCAAGTTCGCTCTGAGG, and CTTGTAGTAAATGTTGGCAGGACT, and the reaction yielded a 500 bp band for the wild type allele and a 400 bp band for the mutant allele.

### 3. Results

We recorded and characterized the ABR of *EphA4* and *ephrin-B2* mutant mice, aged postnatal day 18–20, and compared them to wild type controls. An example ABR is shown in Fig. 1a. Recordings were 10 ms long and included a 2 ms pre-stimulus period. There were typically 4 to 5 waves in each 10 ms trace, as reported previously by Song et al. (2006). The first three waves were termed P1, P2, and P3.

ABR thresholds were higher in both *EphA4* and *ephrin-B2* mutant mice than in wild type mice. Representative examples of ABR traces for genotypes used in the study are shown in Fig. 1a–d. Data were collected every 5 dB between 20 and 60 dB to make a precise determination of

threshold. Both the *EphA4* heterozygote (*EphA4*<sup>+/-</sup>) and homozygote (*EphA4*<sup>-/-</sup>) mice required higher dB SPL to elicit a recognizable response (*EphA4*<sup>+/+</sup>: 31.5 ± 1.26 dB SPL, *n* = 17; *EphA4*<sup>+/-</sup>: 41.6 ± 3.89 dB SPL, *n* = 15; *EphA4*<sup>-/-</sup>: 55.0 ± 2.98 dB SPL, *n* = 10; ANOVA, *p* < 0.05; Fig. 2a). Thus, the threshold value for *EphA4*<sup>-/-</sup> mice was 75% higher than wild type. Thresholds for *ephrin-B2*<sup>lacZ/+</sup> mice were also significantly elevated, with a mean value 20% higher than wild type controls (*ephrin-B2*<sup>lacZ/+</sup>: 59.6 ± 2.36 dB SPL, *n* = 13 vs. *ephrin-B2*<sup>+/+</sup>: 49.6 ± 1.74 dB SPL, *n* = 13; *t*-test, *p* < 0.05; Fig. 2b).

Further analysis of ABR traces revealed that individual waves were also different in some genotype groups (Fig. 3). Measures of amplitude show that P1 was 54% smaller in *EphA4*<sup>-/-</sup> mice (0.564 ± 0.09 μV) when compared to wild type controls (1.228 ± 0.14 μV), and P2 amplitude was 56% smaller (0.363 ± 0.04 μV vs. wild type = 0.817 ± 0.11 μV, ANOVA, *p* < 0.05; Fig. 3a). While P1 amplitude was 38% smaller in *ephrin-B2*<sup>lacZ/+</sup> (0.620 ± 0.09 μV) as compared to wild types (0.994 ± 0.08 μV, *t*-test, *p* < 0.05), P2 amplitude was not significantly different (*ephrin-B2*<sup>lacZ/+</sup>, 0.575 ± 0.08 μV vs. wild type, 0.702 ± 0.07 μV, *t*-test, *p* > 0.05; Fig. 3c).

Measures of latency to waveform peak revealed that P1 and P2 were not different from wild type (1.72 ± 0.0448 ms), for either *EphA4*<sup>+/-</sup> (1.72 ± 0.0398 ms) or *EphA4*<sup>-/-</sup> (1.85 ± 0.079 ms) mice (ANOVA, *p* < 0.05; Fig. 3b). Interestingly, *ephrin-B2*<sup>lacZ/+</sup> mice showed a shorter P2 latency (2.53 ± 0.058 ms vs. wild type, 2.79 ± 0.080 ms; Fig. 3d). However, P3 latency was significantly longer in both *EphA4*<sup>-/-</sup> (4.42 ± 0.178 ms vs. wild type, 3.71 ± 0.172 ms, ANOVA, *p* < 0.05; Fig. 4a) and *ephrin-B2*<sup>lacZ/+</sup> mice (4.07 ± 0.164 ms vs. wild type, 3.57 ± 0.11 ms, *t*-test, *p* < 0.05; Fig. 4b). While the peak maxima were identifiable for these relative measures of latency, measures of P3 amplitude were not recorded as the peaks were frequently merged with adjacent waveforms.

### 4. Discussion

*EphA4* and *ephrin-B2* mutant mice display an altered ABR in comparison to wild type controls, in measures of magnitude and timing. Hearing thresholds were significantly elevated for both homozygous and heterozygous mice, and a concurrent decrease in P1 amplitude was found in both mutants. P2 amplitude was also significantly decreased in *EphA4* mutants, while *ephrin-B2* mutants showed a shorter latency for P2 without any change in amplitude. Both mutants also displayed a longer latency of the P3 wave.

Several functions of Eph proteins might account for the changes we observed in the ABR in mutant mice. Our measurements of reduced amplitude of P1 and P2 waves in *EphA4* mice and reduced P1 wave amplitude in *ephrin-B2*<sup>lacZ/+</sup> mice suggest an auditory nerve and/or cochlear nucleus defect. The expression of *EphA4* in the cochlea

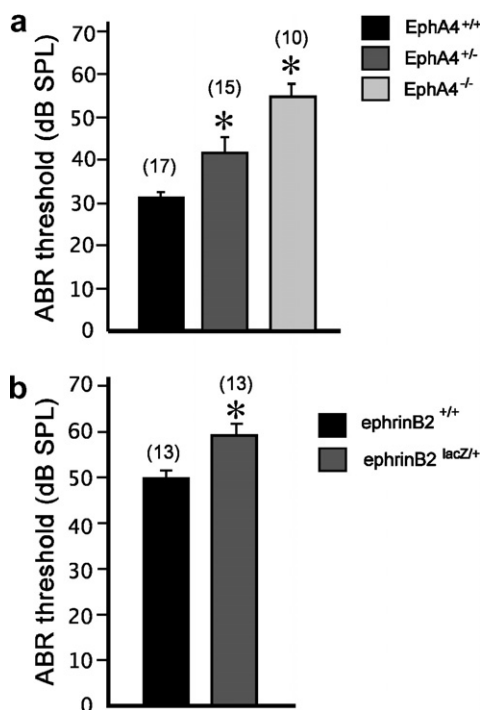


Fig. 2. Measurement of ABR threshold in all genotypes. Both *EphA4*<sup>+/-</sup> and *EphA4*<sup>-/-</sup> mice show a significantly increased ABR threshold as compared to wild type (a), and the mutant value is 75% higher than control. The *ephrin-B2*<sup>lacZ/+</sup> mice also shows a 20% elevated ABR threshold as compared to wild type (b). Numbers of animals for each group are indicated in parentheses.

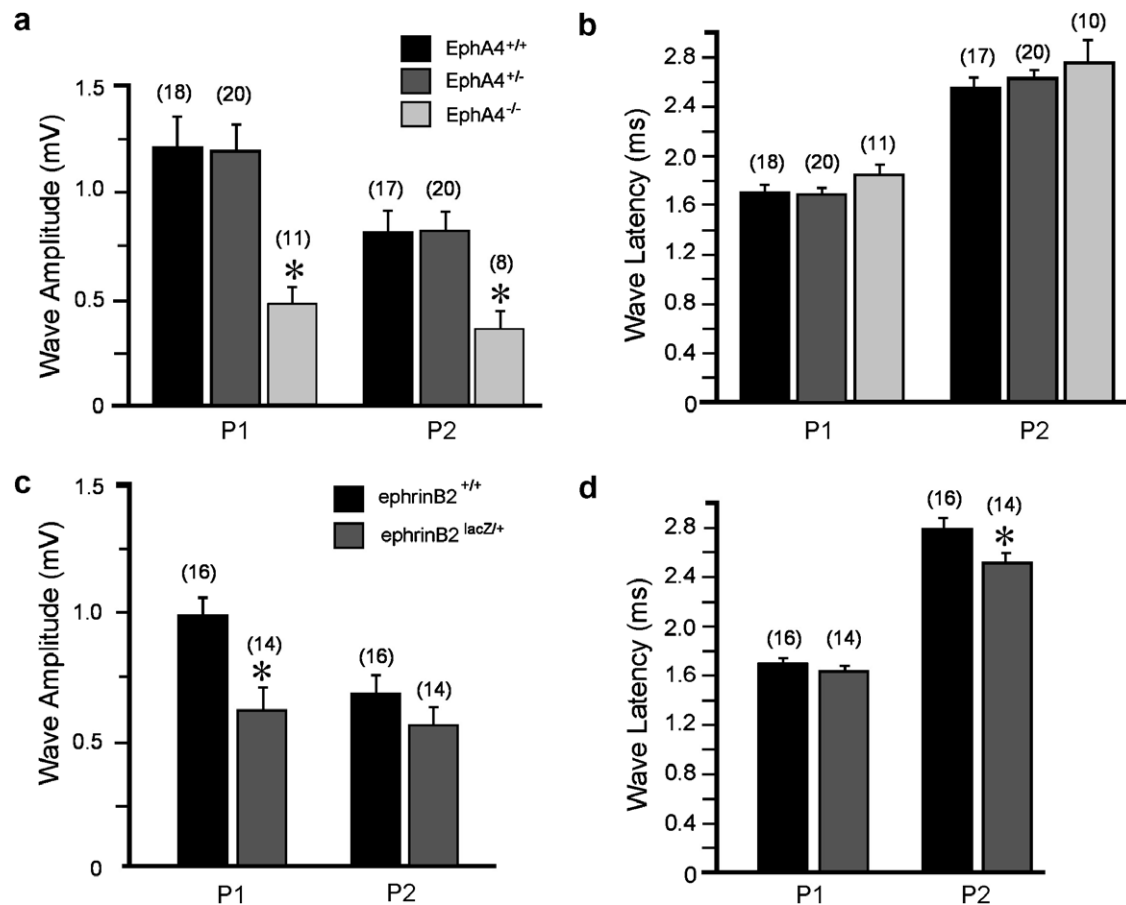


Fig. 3. A comparison of amplitude and latency measures in all genotypes. *EphA4* genotype key applies to (a) and (b) and *ephrin-B2* genotype key applies to (c) and (d). P1 and P2 showed decreased amplitude in *EphA4*<sup>-/-</sup> mice (a), whereas there was no difference in latency for either peak (b). *ephrin-B2*<sup>lacZ/+</sup> mice show a decreased amplitude in P1 (c) and a decreased latency for P2 (d).

(van Heumen et al., 2000) suggests that the change in the ABR may originate peripherally, at least in part. Mutations in some EphB genes alter cochlear function as measured by DPOAEs (Howard et al., 2003), but the specific effect of EphA4 or ephrin-B2 mutation has not been reported. Both neural and non-neural regions of the cochlea express Eph proteins (Pickles, 2003), suggesting that the functions of these proteins may also include structural development (Pickles et al., 2002) and maintenance of the cochlea. In addition, Eph proteins may regulate ion concentrations essential to function in the ear (Dravis et al., 2007). The diversity of functions for Eph proteins, combined with the distinct expression patterns of Eph family members (Bianchi and Gale, 1998; Pickles, 2003), highlight the fact that these proteins may have multiple, complex interactions that ultimately support normal hearing.

Decreases in P1 and P2 wave amplitudes may be directly related to the observed increase in ABR threshold. These first two peaks show the largest amplitudes in the mouse ABR trace, and therefore define the emergence of the peaks from baseline, which is the appearance of the trace at threshold. Previous work has suggested that P1 and P2 reflect activation of the auditory nerve and cochlear

nucleus, respectively (Møller and Jannetta, 1985). Our findings of decreased amplitude in both peaks in the *EphA4*<sup>-/-</sup> mouse may reflect a significant role for this receptor tyrosine kinase in the auditory nerve–cochlear nucleus region. EphA4 is expressed in both the dorsal and ventral cochlear nucleus, and measures of tone-evoked activation of the immediate early gene *c-fos* have shown that the magnitude of activation is significantly decreased in the *EphA4*<sup>-/-</sup> dorsal cochlear nucleus (DCN) as well as medial nucleus of the trapezoid body (MNTB) (Miko et al., in press). Together with the ABR result presented here, there is strong evidence that EphA4 protein has a pronounced effect on the magnitude of evoked activation in auditory brainstem.

The 20% elevation of threshold and 38% reduction of P1 amplitude in ephrin-B2 mutants may also reflect a poorly developed auditory nerve–cochlear nucleus junction. Developing mouse spiral ganglion cells express ephrin-B2 during development of cochlear innervation (Bianchi and Gray, 2002; Pickles, 2003), and cochlear nucleus neurons also express ephrin-B2 during early postnatal development (Miko et al., in press). Studies of adult cochlear nucleus show that ephrin-B2 is also abundant in both dorsal and ventral cochlear nucleus, as well as the superior olive and



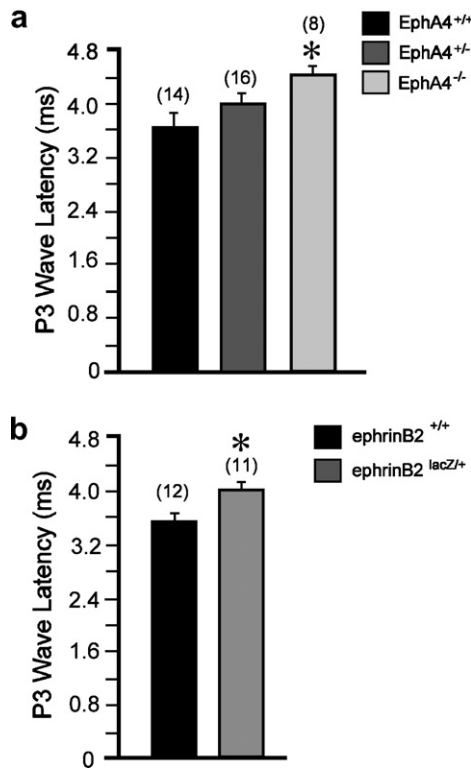


Fig. 4. The latency to P3 peak was longer in *EphA4*<sup>-/-</sup> (a) and *ephrin-B2*<sup>lacZ/+</sup> (b) mice, as compared to matched wild types.

emanating lemniscal pathways. Our previous observation that ephrin-B2 deficiency increases the spread of tone-evoked activation in the dorsal cochlear nucleus *without* decreasing magnitude of activation (number of cells) (Miko et al., *in press*) may corroborate the decreases in P1 amplitude presented here. Thus, the voltage could be attenuated as a result of the same current spread over a larger volume of tissue. However, the use of higher impedance electrodes than those used in the present study would be necessary to explore the relationship between current magnitude and spread of activation over a smaller volume of tissue the size of cochlear nucleus.

The significantly delayed P3 latency in both *EphA4* and *ephrin-B2* mutants may reflect abnormality in the superior olivary complex. Previous work in the guinea pig brainstem has shown that a lesion at the ventral midline that resects the olivary nuclei causes a complete loss of P3, and suggests that the superior olivary complex contributes to this wave of the ABR (Wada and Starr, 1989). Our findings of increased P3 latency in the mutant animals may reflect a processing defect in this part of the brainstem. This observation is consistent with our finding that *EphA4* and *ephrin-B2* are expressed in the olivary nuclei and that tone-evoked activation of *c-fos* in the MNTB is reduced in both mutants (Miko et al., *in press*). A slower neural conduction time through the auditory pathway may be an indication of the integrity of myelin, as increases in ABR peak latencies have been observed in humans with multiple sclerosis (Keith et al., 1987). Interestingly, Eph

proteins are known to interact with myelin through neuroglial interactions (Benson et al., 2005; Goldshmit et al., 2006; Liu et al., 2006). Furthermore, the ephrin-B2 ABR shows an intriguing shorter P2 latency coinciding with a longer P3 latency, which suggests that the weight of this impairment may indeed be in the processing between cochlear nucleus and the superior olivary complex.

Although the P18–20 mouse shows an adult-like ABR waveform (Song et al., 2006), there is evidence that protein expression and synaptic transmission are not fully mature in brainstem auditory structures until after P25 (Blaesse et al., 2006; Friauf et al., 1997; Happe and Morley, 2004). However, we focused on P18–20 because at this age responses to click stimuli are adult-like in the cochlear nerve (Sanes and Constantine-Paton, 1985), and the ABR has matured to include all peaks and latencies present in the adult waveform (Song et al., 2006), yet this age is young enough to minimize the effect of age-related hearing loss, which can occur quite early in the C57/Bl6 strain. The present findings involving Eph receptor impact on function may eventually be compensated for by later ages. To identify any compensation would require ABR testing at multiple time points beyond the one tested here. Above all, deficits seen at P18–20 can have a long-term impact, as they would be present at weaning age, when the mouse uses its hearing to survive independent of the breeding group.

It is important to note that differences in ABR threshold were also apparent between the wild type of each mouse line, which shows that the impact of background strain can be a confounding influence. The definition of normal ABR thresholds were always made within background strain, and the results shown here emphasize the importance of comparing mutant mice with wild type littermates, as strain differences may impact results achieved with knockout mice.

ABR measurements have proven to be a robust method for analyzing the effects of genetic mutations on hearing in mice, both in mutagenesis screens (Kermany et al., 2006) and in the evaluation of candidate genes (Abraira et al., 2007; Blauwkamp et al., 2007; Polley et al., 2006). In these studies, the candidate genes are transcription factors or morphogens with identified roles in nervous system development. Similarly, the ABR data presented here reveal the significant impact of *EphA4* and *ephrin-B2* mutations on the effectiveness of auditory processing. Together with their previously established role as axon guidance molecules, these Eph proteins impact both anatomical and functional aspects of the auditory neural circuit. These abnormalities underscore the importance of Eph family proteins in the formation of normal auditory function.

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