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ScienceDirect
Hearing Research 235 (2008) 39–46
Hearing Research
www.elsevier.com/locate/heares

Research paper

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

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Received 9 August 2007; received in revised form 20 September 2007; accepted 21 September 2007
Available online 29 September 2007

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$^{-/-}$ mice was 75% higher than that of controls. Waveform amplitudes of peak 1 (P1) were 54% lower in EphA4$^{-/-}$ mice than in controls. The peripheral hearing thresholds in *ephrin-B2*$^{lacZ/+}$ mice were also elevated, with a mean value 20% higher than that of controls. These *ephrin-B2*$^{lacZ/+}$ 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.

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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doi:10.1016/j.heares.2007.09.003

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 β-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-β-galactosidase fusion protein in which the cytoplasmic domain of ephrin-B2 has been deleted. Within this strain, ABR measurements were performed on *ephrin-B2*$^{+/+}$ or *ephrin-B2*$^{lacZl+}$ mice, as the *ephrin-B2*^{lacZllacZ} 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 β-galactosidase, which is inserted downstream of the *EphA4* promoter region. ABR measurements were performed on wild type (*EphA4*$^{+/+}$), heterozygous (*EphA4*$^{+/-}$), and homozygous (*EphA4*$^{-/-}$) 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 μs 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