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Auditory Brainstem Responses of ephrin-A2^{-/-}, ephrin-A5^{-/-}, and ephrin-A2A5^{-/-} mice

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Abstract

Eph receptors and ephrin ligands are large families of cell-surface proteins which have established roles in axonal growth and guidance. These are well characterised in the visual and somatosensory systems but are less well documented in the auditory pathway. We examined the possible functional role of two ephrin genes (ephrin-A2 and ephrin-A5) in the auditory system by measuring auditory brainstem responses (ABRs) to tone bursts from 6-30 kHz in ephrin-A2^{-/-}, ephrin-A5^{-/-} and ephrin-A2A5^{-/-} (knockout) mice. At high frequencies, ephrin-A2A5^{-/-} mice exhibited thresholds that were significantly lower than wild-type by approximately 20 dB, suggesting ephrin-A2 and ephrin-A5 may have frequency-specific effects in the auditory system. There were also alterations in ABR wave peak amplitudes that were specific to each mouse strain that suggested both peripheral and central involvement of EphA-ephrin-A signalling in auditory function.

1. Introduction

The Eph tyrosine kinase receptor family of proteins and their ligands, the ephrins [Klein, 2004; Uziel et al., 2006] have been implicated in the development of connectivity within the somatosensory system [Prakash et al., 2000; Uziel et al., 2006; Uziel et al., 2002], visual system [Triplett and Feldheim, 2012], hippocampus [Gao et al., 1999; Gao et al., 1998; Martínez et al., 2005; Otal et al., 2006] and in the auditory system [Miko et al., 2007]. These proteins generate and maintain connections by acting as attractive and/or repulsive guidance cues for axonal growth cones [Mellitzer et al., 2000]. Furthermore, the receptors and ligands are commonly expressed as complementary gradients across the connected regions, underpinning highly organised topographic representations of sensory input [Goodhill and Richards, 1999].

Among the many ephrin ligand family members, ephrin-A2 and ephrin-A5 have particularly well documented roles in visual system function and development [Feldheim et al., 2000; Haustead et al., 2008; McLaughlin et al., 2003; Wilks et al., 2010]. They are essential in guiding the formation of topographically accurate connections between visual nuclei [Brown et al., 2000]; removal of ephrin-A2 and ephrin-A5 results in increased numbers of projections and synapses in visual regions of the brain such as the superior colliculus [Feldheim et al., 2000; Haustead et al., 2008; Triplett et al., 2009; Wilks et al., 2010] and visual cortex [Cang et al., 2005], resulting in visual behavioural deficits [Haustead et al., 2008]. Similarly, removal of ephrin-A5 results in abnormal topography in somatosensory regions of the brain [Prakash et al., 2000; Uziel et al., 2006; Uziel et al., 2002; Vanderhaeghen et al., 2000]. However, few studies have investigated the functional role of these proteins in the auditory system.

During various times in development and adulthood, ephrin-A2 and ephrin-A5 are expressed in several parts of the cochlea and auditory nerve (AN) **including the cochlea connective tissue, cochlear neurons, supporting cells and outer hair cells** [Bianchi and Gale, 1998; Bianchi and Liu, 1999; Defourny et al., 2013; Lee and Warchol, 2005; Pickles, 2003; Pickles et al., 2002; Saeger et al., 2011; Siddiqui and Cramer, 2005]. **Ephrin-A2 and ephrin-A5 are also expressed in more central auditory structures such as the inferior colliculus and medial geniculate body** [Lyckman et al., 2001; Zhang et al., 1996].

However, the functional and guidance roles of these proteins remain largely unexplored in the auditory pathway. Recently, ephrin-A5^{-/-} mice have been shown to have ectopic projections in the cochlea, with type

1 afferent fibres projecting to outer hair cells instead of inner hair cells [Defourny et al., 2013]. The same study used **auditory brainstem responses (ABRs)** and showed normal thresholds and reduced suprathreshold amplitudes to click stimuli in ephrin-A5^{-/-} mice but the sensitivity to individual frequencies was not assessed.

The present study assessed the functional effects of ephrin-A2 and ephrin-A5 single and double knock-out on the auditory thresholds of mice by recording auditory brainstem responses. We analysed thresholds and amplitudes of individual ABR components across a wide range of audible sound frequencies to gain insight into the potential roles of these proteins in regulating connections between auditory brainstem regions and generating tonotopic maps. Overall our data suggest that ephrin-A2 and ephrin-A5 play a role in the development of the auditory system in the cochlea, auditory nerve and/or brainstem auditory nuclei.

2. Methods

Four strains of mice were used in the experiment; WT C57Bl/6J (N=18, age = 14.2 ± 4.8 weeks), ephrin-A2^{-/-} (N=17, age = 19.9 ± 11.6 weeks), ephrin-A5^{-/-} (N=9, age = 22.9 ± 6.0 weeks) and ephrin-A2A5^{-/-} (N=16, age = 22.6 ± 11.0 weeks). The knockout mice of the same background strain as wild-type (C57Bl/6J) and have been backcrossed onto this strain for over 15 generations. It was not feasible to use WT littermates because of the low probability of generating useful single and double knockout homozygote mice from double heterozygote parents. Mice were bred at the University of Western Australia and genotyped at weaning as previously described [Haustead et al., 2008] (ephrin-A2: 5'_CCG CTT CCT CGT GCT TTA CGG TAT C3'_; 5'_ATA CCG TGG AGG TGA GCA TC3'_; 5'_AAA AGG GGG TGA AGA GTT GG3'_; ephrin-A5: 5'_TCC AGC TGT GCA GTT CTC CAA AAC A3'_; 5'_ATT CCAGAGGGGTGA CTA CCA CAT T3'_; 5'_AGC CCA GAA AGC GAA GGA GCA AAG C3'). Animals were anaesthetized with 10 mg/kg xylazine and 70 mg/kg ketamine (i.p.) and were placed on a heating pad at 39°C and wrapped in bubble wrap for insulation for the duration of the auditory brainstem recordings. After recordings were complete, animals were euthanized with intraperitoneal injection of 0.1 ml Lethobarb (325 mg/ml sodium pentobarbitone, Virbac animal health). All procedures were approved by the University of Western Australia Animal Ethics Committee (approval number: 03/100/1020).

Recordings took place in a sound-attenuated room. Auditory brainstem responses were evoked using tone bursts of 5 ms duration with a 1 ms rise-fall-time presented at 10 bursts/sec. The sound was generated by custom made software (Neurosound, MI Lloyd). The output had a 96 kHz sampling rate with optical output from a RME DIGI 9636 sound card connected to an RME AD-8 DS AD/DA converter (RME Intelligent Audio Solutions, Germany). The output of this was connected to a high voltage power supply connected to a reverse-driven ½ inch condenser microphone (Bruel and Kjaer type 4134). A cone was attached to the microphone, and the tip of the cone was placed under visual guidance using a surgical microscope so as to just touch the inner edge of the tragus of the left ear pointing towards the ear canal. The sound pressure level (SPL) was calibrated using a Bruel and Kjaer ¼ inch condenser microphone placed at

the approximate location of the eardrum. Absolute calibration was provided by a Bruel and Kjaer pistonphone (94 dB SPL at 1000 Hz).

Recording electrodes were insulated silver wires inserted subdermally via a 23^{3/4} gauge needle with the negative wire next to the pinna above the left mastoid, the positive wire on the vertex and the ground at the base of the tail. The electrodes were connected to a DAM 50 differential amplifier (World Precision Instruments) with x1000 gain and with a 300-3000 Hz band pass filter. The responses were acquired by a Powerlab/4ST (AD Instruments) which was then linked via USB to a computer running Scope software (AD Instruments). The response was digitised with a sampling rate of 40 kHz, with 2 ms of sampling before tone onset and 10 ms after and was averaged over 400 stimulus presentations.

Thresholds were assessed at 6, 12, 18, 24 and 30 kHz, with 12 kHz presented first followed by the other frequencies in pseudorandom order. Stimuli were first presented at 10 dB attenuation (73 dB SPL) followed by increasing attenuation in 10 dB steps until the peaks were no longer visible. The SPL was then increased by 5 dB to obtain an estimated threshold to within 5 dB. The threshold was taken to be the average dB level between these descending and ascending estimates

Statistical analysis was performed by GraphPad Prism (version 5.04, GraphPad Software Inc.) or RStudio (version 0.97.316) with R (version 2.15.2) and packages stats (version 2.13.1) and ez (version 3.0-0). Repeated measures analysis was used when applicable, and *post-hoc* tests compared the knock-out groups to wild-type, using exact Holm-Bonferroni correction. If one factor was determined not to have a main effect from a 2-way ANOVA the data were pooled, and analysed with a 1-way ANOVA and post-hoc pair-wise comparisons. Averaged data are presented as Means $\pm$ SEMs. We confirmed that age had no effect on threshold using linear regression for hearing threshold for each genotype and frequency assessed (all $p > .05$). **Furthermore, the background strain shows only late onset hearing loss at an age older than our animals** [Hunter and Willott, 1987; Keithley et al., 2004; Zheng et al., 1999].

3. Results

In order to investigate a possible frequency-specific effect of ephrin-A2 and/or ephrin-A5 knockout on hearing, **ABRs** were recorded from each group at a range of frequencies. Example traces are shown in Figure 1. The waveforms produced were characterised by 5 distinct waves which using common convention were designated Wave I to V (Figure 1). A value for threshold was determined offline at each frequency. Following threshold determination, latencies and amplitudes of positive peaks corresponding to Wave I to V were designated as P1 to P5. For each peak, amplitudes were calculated as the maximum peak value – lowest trough value of each wave, and latencies as the time of the maximum amplitude of the corresponding peak. We analysed each wave separately because the different waves reflect evoked signals from different parts of the auditory pathway [Achor and Starr, 1980; Møller and Jannetta, 1983; Wada and Starr, 1983a; Wada and Starr, 1983b; Wada and Starr, 1983c].

Figure 1. Roughly Here

3.1 ABR thresholds

Figure 2 shows thresholds for all genotypes and all frequencies. An omnibus ANOVA (Genotype x Frequency) was performed which showed there was a highly significant main effect of genotype ($F_{(3,204)} = 23.98$, $p < 0.0001$) and interaction of genotype and frequency ($F_{(3,204)} = 4.98$, $p < 0.01$). There was no main effect of frequency ($F_{(1,204)} = 2.00$, $p = 0.16$). Planned one-way ANOVAs were performed within each frequency. There were no significant differences between genotypes at the two lowest frequencies, 6 kHz ($F_{(3,38)} = 0.75$, $p = 0.53$) and 12 kHz ($F_{(3,41)} = 2.07$, $p = 0.12$). However, separate ANOVAs at individual frequencies indicated significant main effects of genotype at the higher frequencies, 18 kHz ($F_{(3,40)} = 6.90$, $p < 0.001$), 24 kHz ($F_{(3,38)} = 3.38$, $p < 0.0001$) and 30 kHz ($F_{(3,35)} = 7.51$, $p < 0.001$). As shown in Figure 2, *post-hoc* comparisons revealed that ephrin-A2A5^{-/-} mice had lower (more sensitive) thresholds than the WT controls (18kHz, $p < 0.001$; 24kHz, $p < 0.0001$; 30kHz, $p < 0.001$). There was a similar trend in the single knockout ephrin-A2^{-/-} mice, but unlike ephrin-A2A5^{-/-} the decrease in threshold reached significance only at 24 kHz ($p < 0.01$) and 30 kHz ($p < 0.05$), but not 18kHz ($p > 0.05$). Thus, ephrin-A2A5^{-/-}, and to a lesser

extent ephrin-A2^{-/-} mice, had a lower threshold to high frequency stimuli compared to WT. Ephrin-A5^{-/-} mice showed no significant alterations in threshold compared to WT (p values all > 0.05).

Figure 2. Roughly Here

3.2 ABR Latencies

Following threshold determination the ABR waveforms were analysed to determine the latencies of each peak at 40 dB above threshold for each frequency (Figure 3). A 2-way ANOVA (Genotype x Frequency) showed that there was a significant effect of frequency for peak 1 latency ($F_{(4,166)} = 8.08$, $p < 0.0001$), with latency decreasing with increasing frequency, consistent with the known mechanical and morphological properties of the cochlea [Neely et al., 1988]. There was also a significant effect of genotype ($F_{(3,166)} = 5.39$, $p < 0.01$), though pair-wise post-hoc tests did not reveal source of these differences. For all other peaks, P1 latency was subtracted from the peak latency in order to provide an estimate of neural conduction time. Peaks 2, 3 and 5 showed a significant effect of genotype (P2, $F_{(3,162)} = 4.75$, $p < 0.01$; P3, $F_{(3,162)} = 3.83$, $p < 0.05$; P5, $F_{(3,162)} = 4.96$, $p < 0.01$). Peak 4 was not significant for genotype ($F_{(3,162)} = 2.57$, $p = 0.06$). None of the peaks 2 to 5 had significant effects of frequency (P2, $F_{(3,162)} = 0.12$, $p = 0.98$; P3, $F_{(4,162)} = 1.19$, $p = 0.32$; P4, $F_{(4,162)} = 0.88$, $p = 0.48$; P5, $F_{(4,162)} = 0.30$, $p = 0.87$).

Because there were no main effects of frequency on P2 to P5 latency, data was pooled across frequencies for each genotype (Figure 3, bars) and peak. ANOVAs were performed on the pooled data which showed that there was a significant effect of genotype at each peak (P2, $F_{(3,178)} = 4.95$, $p < 0.01$; P3, $F_{(3,172)} = 2.80$, $p < 0.05$; P4, $F_{(3,178)} = 3.14$, $p < 0.05$; P5 $F_{(3,178)} = 5.76$, $p < 0.001$). As shown in Figure 3, there were post-hoc differences ephrin-A5^{-/-} mice which had prolonged latencies from P2 to P5 (all $p < 0.05$). All knock-out groups showed prolonged latencies for P5 (all $p < 0.05$).

Figure 3. Roughly Here

To provide more information about latency, each animal's responses were determined from 33-73 dB SPL in response to 18 kHz tone bursts. The selected SPL range allowed suprathreshold analysis for all genotypes. We chose 18kHz because this was the most sensitive part of the audiogram at which genotype effects were evident (Figure 4). As shown in Figure 4, P1 latency had a significant effect of genotype ($F_{(3,191)} = 6.34$, $p < 0.001$) and sound intensity ($F_{(4,191)} = 5.10$, $p < 0.001$).

Figure 4. Roughly Here

For all other peaks, P1 latency was subtracted from the peak latency (Figure 4B – 4E). The P2-P1 latency was characterised by flat input-output curves and no significant differences, suggesting that auditory nerve conduction velocity (Wave I to wave II) was normal in all genotypes (Genotype, $F_{(3,193)} = 0.34$, $p = 0.80$; Sound Intensity, $F_{(4,193)} = 1.04$, $p = 0.39$; Interaction, $F_{(12,193)} = 0.68$, $p = 0.77$). There was a significant effect of genotype for P3, P4 and P5 latency relative to P1, but no effect of sound intensity or interaction effects (P3-P1: genotype, $F_{(3,193)} = 4.46$, $p < 0.01$; sound intensity, $F_{(4,193)} = 0.35$, $p = 0.85$; interaction, $F_{(12,193)} = 0.50$, $p = 0.91$; P4-P1: genotype, $F_{(3,203)} = 3.4$, $p < 0.05$, sound intensity, $F_{(4,203)} = 0.038$, $p = 0.997$; interaction, $F_{(12,203)} = 0.13$, $p = 0.9998$; P5-P1: genotype, $F_{(3,183)} = 7.02$, $p < 0.001$ sound intensity, $F_{(4,183)} = 0.37$, $p = 0.83$; interaction, $F_{(12,183)} = 1.16$, $p = 0.31$). This allowed us to pool the data across sound intensities. For P3-P1 and P5-P1, one-way ANOVA showed significant differences between genotypes (P3-P1, $F_{(3,209)} = 4.67$, $p < 0.01$; P5-P1, $F_{(3,209)} = 4.381$, $p < 0.01$), and pair-wise comparisons showed that this difference was due to an elevation in ephrin-A5^{-/-} latency ($p < 0.05$) for both peaks. Despite a significant effect of genotype in pooled P4-P1 latencies ($F_{(3,219)} = 3.68$, $p < 0.05$), pair-wise comparisons showed only a trend for reduced latency in ephrin-A2A5^{-/-} mice.

3.3 ABR Amplitudes

The amplitudes of each wave were determined at 40dB above threshold at each frequency (Figure 5). There was a significant main effect of genotype for all peak amplitudes (P1, $F_{(3,140)} = 17.33$, $p < 0.0001$; P2, $F_{(3,140)} = 8.52$, $p < 0.0001$; P3, $F_{(3,140)} = 6.58$, $p < 0.001$; P4, $F_{(3,140)} = 9.63$, $p < 0.0001$; P5, $F_{(3,140)} = 8.52$, $p < 0.0001$). There was a significant effect of frequency for P2 amplitude (P2, $F_{(4,140)} = 2.53$, $p < 0.05$), but this was not found with other peaks (P1, $F_{(4,140)} = 1.98$, $p = 0.10$; P3, $F_{(4,140)} = 0.79$, $p = 0.54$; P4, $F_{(4,140)} = 1.29$, $p = 0.28$; P5, $F_{(4,140)} = 2.14$, $p = 0.08$). There were no significant interactions between genotype and frequency (P1, $F_{(12,140)} = 0.51$, $p = 0.91$; P2, $F_{(12,140)} = 0.21$, $p = 0.998$; P3, $F_{(12,140)} = 0.77$, $p = 0.68$; P4, $F_{(12,140)} = 0.37$, $p = 0.97$; P5, $F_{(12,140)} = 1.09$, $p = 0.37$). Because there was no main effect of frequency for P1, P3, P4, and P5 data for these peaks was pooled and analysed by 1-way ANOVAs. There was significant difference between

genotypes for each of these peaks (P1, $F_{(3,156)} = 18.90$, $p < 0.0001$; P3, $F_{(3,156)} = 7.56$, $p < 0.0001$; P4, $F_{(3,156)} = 11.14$, $p < 0.0001$; P5, $F_{(3,156)} = 9.86$, $p < 0.0001$). The analysis showed that there was a significant increase in ephrin-A2^{-/-} ($p < 0.001$) and decrease in ephrin-A5^{-/-} ($p < 0.01$) P1 amplitude. Ephrin-A2A5^{-/-} showed increased amplitudes for P4 and P5 ($p < 0.01$). Ephrin-A5^{-/-} also showed increased amplitude for P4 ($p < 0.01$).

Figure 5. Roughly Here

In order to investigate whether changes in amplitude of later peaks were related to changes in earlier peaks, we analysed the amplitude of each peak relative to P1 for responses to 18kHz tone pips 40dB above threshold. The amplitude ratios of P2, P3 and P4 relative to P1 were not significant (data not shown, P2, $F_{(3,33)} = 1.23$, $p = 0.31$; P3, $F_{(3,34)} = 0.70$, $p = 0.56$; P4, $F_{(3,34)} = 1.97$, $p = 0.14$). The only significant difference occurred in the P5/P1 ratio (Figure 5F; ANOVA $F_{(3,34)} = 4.14$, $p < 0.05$) which was due to significantly elevated ratio in ephrin-A2A5^{-/-} mice ($p < 0.05$).

Changes in peak amplitudes were further investigated at a range of sound pressures between 33 to 73dB SPL in response to 18kHz tone bursts (Figure 6). There was a main effect of genotype for each wave peaks (P1, $F_{(3,211)} = 17.20$, $p < 0.0001$; P2, $F_{(3,211)} = 16.42$, $p < 0.0001$; P3, $F_{(3,211)} = 6.034$, $p < 0.001$; P4, $F_{(3,211)} = 16.98$, $p < 0.0001$; P5, $F_{(3,211)} = 33.03$, $p < 0.0001$) and for sound intensity peaks (P1, $F_{(4,211)} = 21.52$, $p < 0.0001$; P2, $F_{(4,211)} = 16.05$, $p < 0.0001$; P3, $F_{(4,211)} = 12.46$, $p < 0.0001$; P4, $F_{(4,211)} = 10.73$, $p < 0.0001$; P5, $F_{(4,211)} = 14.56$, $p < 0.0001$), but never an interaction effect (P1, $F_{(12,211)} = 1.37$, $p = 0.18$; P2, $F_{(12,211)} = 1.56$, $p = 0.11$; P3, $F_{(12,211)} = 1.07$, $p = 0.39$; P4, $F_{(12,211)} = 0.37$, $p = 0.97$; P5, $F_{(12,211)} = 0.78$, $p = 0.67$), indicating that changes in sound pressure affected all genotypes equally (Figure 6). The differences in amplitude were primarily due to elevation of amplitude in ephrin-A2A5^{-/-} mice, which was consistently found in peaks P2-P5 and was most evident in peak 5. However, peak 1 showed a different pattern: ephrin-A2A5^{-/-} mice had normal amplitudes while ephrin-A2^{-/-} and ephrin-A5^{-/-} mice had elevated and reduced amplitudes respectively. When amplitudes were expressed relative to peak 1, there was a main effect of genotype for each ratio (P2, $F_{(3,211)} = 6.84$, $p < 0.001$; P3, $F_{(3,211)} = 3.88$, $p < 0.01$; P4, $F_{(3,211)} = 8.95$, $p < 0.0001$; P5, $F_{(3,211)}$

= 24.88, $p < 0.0001$), but post-hoc tests showed significance only in the P5/P1 ratio which showed that ephrin-A2A5^{-/-} mice had elevated ratios (Figure 6F). The source of genotype effect for the P2, P3 and P4 ratios was unclear. There were no main effects of sound intensity (P2, $F_{(4,211)} = 0.65$, $p = 0.62$; P3, $F_{(4,211)} = 0.71$, $p = 0.59$; P4, $F_{(4,211)} = 0.37$, $p = 0.83$; P5, $F_{(4,211)} = 0.47$, $p = 0.996$) or any interactions in this data (P2, $F_{(12,211)} = 0.85$, $p = 0.60$; P3, $F_{(12,211)} = 0.32$, $p = 0.99$; P4, $F_{(12,211)} = 0.54$, $p = 0.88$; P5, $F_{(12,211)} = 0.24$, $p = 0.75$).

4. Discussion

In summary, our data suggest that ephrin-A2 and ephrin-A5 play a role in auditory system development and have effects on mature auditory system function. Furthermore, our data indicate that these effects are exerted in both peripheral and central components of the system.

4.1 Ephrin-A2^{-/-} and Ephrin-A2A5^{-/-} Mice show lowered thresholds compared to wild-type mice

Perhaps the most striking finding of this study is that there was a significant increase in sensitivity in hearing thresholds at high frequencies for ephrin-A2^{-/-} and ephrin-A2A5^{-/-} mice. This finding is robust, suggestive of an important role for ephrin-A2 and ephrin-A5 in the auditory brainstem. The difference in threshold is additionally supported by the separation of sound intensity/frequency input-output curves between genotypes. The curves show increased peak amplitudes for ephrin-A2 and more strikingly for ephrin-A2A5^{-/-} mice. Thus for a given sound intensity or frequency the auditory brainstem of these mice generates a larger response, consistent with a lower threshold. The potential sources of these differences is discussed below by looking individual genotypes and effects on individual peaks.

4.2 Ephrin-A5^{-/-} Mice Show Wave Amplitudes and Latencies which reflect alterations in auditory nerve function

Despite the fact that ephrin-A5^{-/-} mice had normal thresholds, the amplitude of wave I was reduced at all intensities, suggesting reduced suprathreshold function of the peripheral auditory nerve [Achor and Starr, 1980; Møller and Jannetta, 1983; Starr et al., 1996; Stockhard and Rossiter, 1977]. **This result has interesting comparisons to human tinnitus sufferers which also show normal thresholds but reduced wave I amplitudes, consistent with reduced primary afferent activity or synchronization [Schaette and McAlpine, 2011].** A reduction in auditory nerve suprathreshold function is supported by the prolonged P2-P1 latencies at 40dB above threshold, which suggests that conduction velocity in the auditory nerve is reduced in ephrin-A5^{-/-} mice. Our results using tone evoked ABRs responses in ephrin-A5^{-/-} mice are

consistent with the ABR findings of Defourney *et al.* [2013], who also demonstrated normal thresholds but reduced suprathreshold amplitudes using click stimuli in the same mouse strain. In development, *in situ hybridization* in the mouse shows that ephrin-A5 is expressed in the developing auditory system during E8.5-E10 [Saeger et al., 2011] and is also expressed in the embryonic statoacoustic ganglion of rat [Bianchi and Gale, 1998]. Most recently ephrin-A5 has been examined in the cochlea, and has been shown to be expressed in outer hair cells [Defourney et al., 2013]. In normal animals, projections of EphA4 positive auditory nerve fibres are restricted to inner hair cells in the cochlea by ephrin-A5 expression in OHCs [Defourney et al., 2013]. When ephrin-A5 is removed, EphA4 positive AN fibres project to OHCs instead of IHCs, resulting in reduced functional synapses at IHCs [Defourney et al., 2013]. Loss of IHC innervation is consistent with wave I reduced amplitudes. The maintained threshold, despite reduction of suprathreshold amplitude, could be explained by the previously described sparing of a small percentage of AN fibres projecting to IHCs [Defourney et al., 2013]. If these spared fibres correspond to the subset of neurons that normally exhibit the lowest thresholds, the ABR threshold may remain the same. It has been previously demonstrated that thresholds are relatively insensitive to loss of neuronal function until relatively large levels of damage [El-Badry and McFadden, 2007; Gu et al., 2012]. Thus ephrin-A5^{-/-} mice may exhibit abnormal hearing but normal thresholds in a similar way to tinnitus sufferers [Schaette and McAlpine, 2011].

It is an unusual finding that there is decreased peripheral AN function (wave I amplitude) but not decreased proximal AN function (wave II input-output amplitude). This suggests that while there is a reduction in input from IHCs to the AN, the inputs to **cochlear nucleus (CN)** from the AN are near normal levels. It is possible that there may also be down-stream effects in addition to those intracochlear changes described by Defourney [2013]. One possible source of changes could be explained by loss of interaction between ephrin-A5 positive AN fibres with EphA7 in the CN [Rogers et al., 1999]. There are many examples that show that ephrin-A5-EphA7 interactions are inhibitory to neurite outgrowth (hippocampus [Gao et al., 1999], somatosensory cortex [Miller et al., 2006]). Assuming that EphA7 and ephrin-A5 interact in a similar way in the CN, removal of ephrin-A5 may result in sprouting of central processes of AN fibres

and increased projections to the CN due to loss of inhibitory interactions with EphA7. This could result in wave II showing normal amplitude despite reduced peripheral activity. The prolonged P2-P1 latency supports the notion of alterations in CN innervation and changes conduction velocity between the proximal auditory nerve and CN.

4.3 Ephrin-A2^{-/-} Mice Show Reduced Auditory Thresholds and Alterations in Wave Amplitudes

Indicating Possible changes in Auditory nerve function

Ephrin-A2^{-/-} mice showed frequency-specific increased sensitivity (reduced thresholds) and increased wave I amplitude. These results suggest that ephrin-A2 may play an important role in peripheral auditory nerve function in the cochlea. **Expression studies show that ephrin-A2 is expressed in the AN and acoustic ganglia [Bianchi and Gale, 1998; Lee and Warchol, 2005; Pickles et al., 2002; Siddiqui and Cramer, 2005] as well as in supporting cells and connective tissue of the cochlea [Pickles et al., 2002]. The most likely binding partner for ephrin-A2 in this system is the EphA4 receptor which is expressed by IHC [Bianchi and Gale, 1998]. To date, the functional consequences of ephrin-A2-EphA4 binding have not been examined in the auditory system, although they are known to bind with high affinity [Flanagan and Vanderhaegen, 1998] Furthermore, studies in the visual system suggest ephrin-A2-EphA4 binding leads to neurite repulsion [Yin et al., 2004], consistent with the largely complementary expression patterns of these proteins in the cochlea [Bianchi and Gale, 1998]. The increased wave I amplitude may therefore be due to a loss of inhibitory interactions between ephrin-A2 and EphA4 receptors, resulting in increased AN innervation of IHCs.** In support of the possibility EphA4 repulsed developing cochlear ganglion neurites, although the role of ephrin-A2 was not assessed [Brors et al., 2003]. The increase in the amplitudes of later peaks is expected to be due to increases in peripheral sensitivity, because amplitude ratios for all other peaks to peak 1 were normal (data not shown). Furthermore, latencies of early these peaks corrected for peak 1 appear normal, suggesting once cochlear effects are considered all other nuclei response reasonably normal.

4.4 Ephrin-A2A5^{-/-} Mice Show Reduced Auditory Thresholds and Increased Amplitudes of Wave II to V

Ephrin-A2A5^{-/-} mice demonstrate reduced thresholds at 18 – 30kHz, which again suggests a frequency-specific increased neuronal sensitivity to sound. This implies that ephrin-A2 and/or ephrin-A5 may play a role in generating topographic maps in the auditory brainstem. However, in contrast to the single knockouts (ephrin-A5^{-/-} and ephrin-A2^{-/-}), ephrin-A2A5^{-/-} mice have normal wave I amplitudes, suggesting overall normal distal AN sensitivity. Strikingly however, ephrin-A2A5^{-/-} mice had elevated amplitudes for peaks 2 (proximal AN) to 5 (inferior colliculus/lateral lemniscus).

The normal wave I amplitude in ephrin-A2A5^{-/-} mice may reflect opposing effects of ephrin-A5^{-/-} (reduced amplitude, reduced IHC synapses) and ephrin-A2^{-/-} (increased amplitude, predicted increased IHC synapses). The increased amplitude of peaks later than wave I may be due to an increase in neuronal responsiveness or recruitment of more postsynaptic neurons downstream of the peripheral AN. Lack of ephrin-A2 and ephrin-A5 could promote exuberant AN projections to the CN due to loss of inhibition with EphA receptors, as described above. The increased amplitudes in waves III and IV (superior olivary complex and lateral lemniscus) could be a consequence of increased wave II (proximal AN and CN).

While all wave amplitudes in ephrin-A2A5^{-/-} were increased, when differences in threshold and peripheral AN activity were taken into account, only wave V (inferior colliculus) showed significantly increased amplitude, implying that the inferior colliculus shows increased responsiveness relative to other auditory structures. In normal development ephrin-A5 is expressed in the inferior colliculus [Zhang et al., 1996] so it would be expected that ephrin-A5^{-/-} mice would have a phenotype involving this structure. The increased ratio of P5 to P1 amplitude suggests that there is an increase in recruitment of neurons between the peripheral AN and inferior colliculus. This was not found in ephrin-A5^{-/-} mice, which may be due to confounding effects of P1 amplitudes and thus peripheral AN activity. It is possible that ephrin-A5 knock-out increases the number or strength of connection between other auditory brainstem nuclei such as the inferior colliculus, whilst decreasing AN function. However the increased connectivity may only be revealed in ephrin-A2A5^{-/-} mice where AN activity is normal.

4.5 Latency Effects are Complex but may be explained by Disordered Connections

The latency input-output curves show that there are significant differences between the genotypes. Overall our latency data suggests that ephrin-A5 and potentially ephrin-A2 have roles in latency, though the precise roles are not clear. Latency effects have previously been observed in the superior colliculus of ephrin-A2A5^{-/-} mice [Rodger et al., 2012] although the mechanism was not explored.

5. Conclusions

In conclusion, our data demonstrate that ephrin-A2 and ephrin-A5 differentially affect auditory brainstem responses in mice. In particular ephrin-A2^{-/-} and ephrin-A2A5^{-/-} mice show reduced thresholds. This does not necessarily mean that ephrin-A2A5^{-/-} mice have superior hearing behaviourally, but only that their threshold estimated electrophysiologically, is better. In addition, auditory changes are frequency-specific, which suggests that ephrin-A2 and ephrin-A5 may have a role in establishing and/or maintaining tonotopic maps in the auditory system as they do in the visual system [Triplett et al., 2009; Wilks et al., 2010] although direct mapping experiments would be needed to confirm this.

Analysis of wave peak amplitudes of the ABR suggests that ephrin-A5 and ephrin-A2 affect cochlear function, in particular, the relative innervation densities of inner and outer hair cells. While the cochlea-dependent effects of ephrin-A5^{-/-} have been studied in detail previously, and provide much insight into ABR abnormalities, the anatomical role of ephrin-A2 in the mature auditory system and the reasons for reduced thresholds of ephrin-A2^{-/-} and ephrin-A2A5^{-/-} remains relatively unknown. The most prominent central effects were observed in the double ephrin-A2A5^{-/-} mice, presumably because of redundancy within the ephrin-A family. For example EphA2 - EphA8 receptors are capable of binding to all ephrin-A proteins [Aoto and Chen, 2007].

The improvement in estimated thresholds nonetheless is a robust finding that is both highly unusual and exciting. If ephrin-A2 and/or ephrin-A5 do restrict connectivity in the auditory system, they may be potential therapeutic targets for promoting repair and/or regeneration. This is an interesting possibility as ephrin-A2 is upregulated during hair cell regeneration, and may play an important role in axon guidance [Lee and Warchol, 2005]. If these proteins could be targeted by antagonistic therapeutics as has been explored elsewhere in the nervous system [Goldshmit et al., 2004; Rodger et al., 2004], there is the

possibility of enhancing the regenerative capacity of the cochlea. It would also be interesting to assess ephrin-A^{-/-} mice for any possible alterations in presbycusis, especially considering the high-frequency enhancement found in our animals.

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8. Figure legends

Figure 1. Example traces of auditory brainstem responses from each genotype from 12kHz tone presentations. The sound pressure level is illustrated on the left hand side of each graphs, and the genotype of the animal is listed below the graph. The wave peaks are illustrated by roman numerals above the first waveform.

Figure 2. Estimated thresholds as determined by ABR at each frequency. ANOVA results are shown above the bars for each frequency. Post-hoc analyses are shown in the line breaks and compare the KO groups to wild-type. Significance values: * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$. Error bars represent SEM.

Figure 3. Wave peak latencies in response to sound 40dB above threshold at each frequency. The raw latency of peak 1 is shown in A). Graphs B) to E) show latencies of peaks 2 to 5 respectively corrected for peak 1 latency. To the right of the line graphs in B) to E) are bar graphs showing the results of pooling data across frequencies. Data was analysed using a 2-way (Genotype X Frequency), regular ANOVAs, and post-hoc analysis used Holm-Bonferroni corrected t-tests comparing Wild-Type to each Knock-Out. Significance: * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$. Error bars represent SEM.

Figure 4. Input-output responses of latency of ABR peaks evoked by 18kHz tone pips. The raw latency of peak 1 is shown in A). Graphs B) to E) show latencies of peaks 2 to 5 respectively corrected for peak 1 latency. To the right of the line graphs in B) to E) are bar graphs showing the results of pooling data across sound intensities. Data was analysed using a 2-way (Genotype X Sound Intensity), regular ANOVAs, and post-hoc analysis used Holm-Bonferroni corrected t-tests comparing Wild-Type to each Knock-Out. Significance: * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$. Error bars represent SEM.

Figure 5. Wave peak amplitudes in response to sound 40dB above threshold at each frequency for each genotype. Wave peak 1 to 5 amplitudes are shown in A) to E) respectively. The amplitude ratio of P5 to P1

is shown in panel F). Graphs A), and C) to E) show bar graphs of the data pooled across frequencies. Data was analysed using a 2-way (Genotype X Sound Intensity), regular ANOVAs, and post-hoc analysis used Holm-Bonferroni corrected t-tests comparing Wild-Type to each Knock-Out. Significance: * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$. Error bars represent SEM.

Figure 6. Input-output responses of amplitude of ABR peaks evoked by 18kHz tone pips. Data from peaks 1 to 5 are shown in figures A) to E) respectively. The amplitude ratio of P5 to P1 is shown in panel F). Data was analysed using a 2-way ANOVA (Genotype X Frequency) for A) to E) and 1-way ANOVA for F). Post-hoc analysis used Bonferroni-corrected t-tests comparing Wild-Type to each Knock-Out. Significance: * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$. Error bars represent SEM.

Wild-Type

Ephrin-A5^{-/-}

Ephrin-A2^{-/-}

Ephrin-A2A5^{-/-}