Research Article

Changes in Cochlear PMCA2 Expression Correlate with the Maturation of Auditory Sensitivity

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ABSTRACT

The plasma membrane Ca^{2+} ATPase 2 (PMCA2) is necessary for auditory transduction and serves as the primary Ca^{2+} extrusion mechanism in auditory stereocilia bundles. To date, studies examining PMCA2 in auditory function using mutant mice have focused on the phenotype of late adolescent and adult mice. Here, we focus on the changes of PMCA2 in the maturation of auditory sensitivity by comparing auditory responses to RNA and protein expression levels in haploinsufficient PMCA2 and wild-type mice from P16 into adulthood. Auditory sensitivity in wild-type mice improves between P16 and 3 weeks of age, when it becomes stable through adolescence. In haploinsufficient mice, there are frequency-dependent loss of sensitivity and subsequent recovery of thresholds between P16 and adulthood. RNA analysis demonstrates that $\alpha\text{-Atp2b2}$ transcript levels increase in both wild-type and heterozygous cochleae between P16 and 5 weeks. The increases reported for the $\alpha\text{-Atp2b2}$ transcript type during this stage in development support the requisite usage of this transcript for mature auditory transduction. PMCA2 expression also increases in wild-type cochleae between P16 and 5 weeks suggesting that this critical auditory protein may be involved in normal maturation of auditory sensitivity after the onset of hearing. We

also characterize expression levels of two long noncoding RNA genes, *Gm15082* (lnc82) and *Gm15083* (lnc83), which are transcribed on the opposite strand in the 5' region of *Atp2b2* and propose that the lnc83 transcript may be involved in regulating $\alpha\text{-Atp2b2}$ expression.

Keywords: *Atp2b2*, hearing, development, cochlea, noncoding RNA

INTRODUCTION

Auditory transduction relies on the development of a complex set of cell types to transform the mechanical movements of the basilar membrane into an electrical signal that can be interpreted by the brain. The organ of Corti, the auditory sensory epithelium of the cochlea, is made of a range of stereotypically defined cell types including nonsensory cells and inner and outer hair cells as well as a variety of supporting cell types which include Deiters' cells, Hensen's cells, and pillar cells that maintain the structural morphology of the epithelium. In combination with the development of specialized cells in the sensory epithelium of the inner ear are corresponding changes in the expression of proteins and transcription factors that eventually determine the physiological function of a particular cell type (Kelley 2006).

It has been well established that PMCA2 is required for auditory transduction in the mature mammalian

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cochlea (Kozel et al. 1998; Street et al. 1998; Yamoah et al. 1998; Dumont et al. 2001; McCullough and Tempel 2004). PMCA2 is a neuronal PMCA isoform that is expressed in spiral ganglia neurons which innervate the hair cells of the inner ear, and is abundantly expressed in the stereocilia bundles of outer hair cells (Yamoah et al. 1998; Dumont et al. 2001; Wood et al. 2004; Chen et al. 2012). Coordinated auditory responses are not detected in mice until around P12 which is commonly denoted as the “onset of hearing” (Mikaelian and Ruben 1965). Expression of PMCA2 mRNA is detected as early as P0 in the rodent cochlea, and strong expression persists in spiral ganglia and outer hair cells into adulthood (Furuta et al. 1998). Concordantly, PMCA2 expression begins to appear at P0 in outer hair cells at the base of the cochlea, reaches the apex by P6, and expression along the tonotopic axis is maintained in stereocilia bundles through P21 (Chen et al. 2012).

These studies suggest that PMCA2 expression is established before the onset of hearing and helps permit the initiation of auditory transduction. However, it is less clear if there are changes in PMCA2 mRNA and protein expression after the onset of hearing and into adulthood. We set out to determine if PMCA2 plays a role in the maturation of auditory transduction immediately after the onset of hearing. In this study, we assess auditory sensitivity and PMCA2 expression in both wild-type and reduced-PMCA2 expressing mice beginning at P16 and show that PMCA2 regulation is dynamic even after the onset of hearing.

METHODS

Animals

The reduced expression strain, $+/\text{dfw}^{i5}$ (Watson and Tempel 2013), and wild-type littermates used in this study ranged in age from P16 to 9 weeks of age. All of the dfw^{i5} mice have been backcrossed into CBA/CaJ (CBA) for 10–13 generations, designated as incipient congenic CBACa.D2(B6)- $\text{Atp2b2}^{\text{dfwi}5}$. The original dfw^{i5} mouse was on a mixed DBA/2J (DBA) and C57BL6/J (B6) background, with the mutation on a DBA allele. Known hearing loss loci in B6 and DBA were crossed out of our dfw^{i5} line by N5. The $+/\text{dfw}^{i5}$ mice and littermate controls used for expression analysis at P16 and P20 were time-mated and tested exactly 4 days apart to be as precise as possible at this early developmental stage. Tissue was collected from $+/\text{dfw}^{i5}$ mice and wild-type littermates at P16, P20, and 5 weeks of age for Atp2b2 and PMCA2 expression analysis. P16 and P20 tissues were collected on the same day as ABR testing in these individuals. Tissue from mice tested at 5 weeks

(P35–P38) was collected between P38 and P48, and for simplicity is referred to as “5-week” tissue, even if it was collected in the sixth post-natal week. Another haploinsufficient allele, $\text{Atp2b2}^{\text{dfw}2J}$, used in this study arose spontaneously in CBy.A/J- Tlc7^{fsn} at the Jackson Laboratory (JAX) and was obtained in 1997. The $+/\text{dfw}^{2J}$ mice have also been backcrossed into CBA and were from generations 18 to 20, designated as congenic strain CBACa.Cby- $\text{Atp2b2}^{\text{dfw}2J}$. Mice are maintained in a backcross to CBA which are obtained from JAX and replaced every three to four generations to retain a stock that is isogenic with CBA at JAX. Animals were kept on a 12-h light/dark cycle and kept in an environment with minimal exposure to noise. All procedures were approved by the University of Washington Institutional Animal Care and Use Committee.

Auditory Testing

All mice were tested for auditory sensitivity using auditory brainstem responses (ABRs) across frequencies ranging from 5.6 to 40 kHz. Mice were anesthetized with a mixture of ketamine (130 mg/kg) and xylazine (10 mg/kg) prior to auditory testing and put into a sound proof box on a heating pad and secured with a bite plate. The system was calibrated at the beginning of each day of testing to confirm the equipment was working properly and that the speaker output was within the normal range. To ensure consistency between recordings at early developmental ages, P16 and 3 weeks, the same calibration file was loaded for all ABR testing of these groups. A series of 350 tones at a given frequency and intensity were administered, and brain responses were recorded with two electrodes placed subcutaneously at the forebrain and hindbrain. A reference electrode was placed on the animal's hindlimb, and electrocardiogram recordings were monitored to ensure an appropriate level of anesthesia throughout the experiment. Tones were 3 ms long with a 1-ms rise/fall $\cos^2$ function (delivered with alternating polarity). Brain waves were recorded for 15 ms following the initiation of each tone with 75 ms spacing between repetitions (13.3 Hz). ABRs are amplified (1,000 $\times$) and filtered (0.3–3 kHz) by a pre-amplifier (P55; Grass Telefactor, West Warwick, RI) and digitized. Threshold at a particular frequency was determined by visual detection as the lowest intensity sound (dB SPL) which evoked a recognizable and reproducible brain wave (at least two out of three trials), usually wave V.

Real-Time PCR

For RNA expression analysis, fresh brain stem or cochlear tissue was stored in *RNAlater* (Qiagen),

equilibrated for 24 h at 4 °C to allow the solution to penetrate the tissue, and transferred to -20 °C for later use. After perfusion of the cochleae with RNAlater, the vestibular portion of the inner ear was removed in cochlear dissections in addition to the removal of as much bone, vasculature, debris, and neuronal tissue as possible. The protocols for RNA isolation, primers, and procedures for qPCR were the same as described previously (Watson and Tempel 2013). Copy numbers for each transcript of interest were normalized to those of reference genes, *Actb* (β -actin) and *Sdha* (succinate dehydrogenase, subunit A). Additional primer sets used to quantify specific *Atp2b2* transcript types, noncoding RNA genes, and *Atp2b4* are as follows: α -*Atp2b2*, 5'-CGGAGTGTGGACTGACAGCA-3' and 5'-GGTTACATCAGAGGCGCCAG-3'; β -*Atp2b2*, 5'-GCTGGCGATTGCCTTAGC-3' and 5'-GAGGAGTGTCCCCAGGAGTG-3'; *Gm15082*, 5'-CAGGCAATGGTAGTTACCCTGTA-3' and 5'-GAAAGCCACTCAGGGAAGTG-3'; *Gm15083*, 5'-CTCATGCTCCTGCTGTGAAG-3' and 5'-GGTTCACCAACTCCCTGAAG-3'; and *Atp2b4*, 5'-TCCCAGTGGCTGAGATTGTG-3' and 5'-TCTTCAGATCATTTCCCTGGATTAG-3'.

Cochlear Westerns

The Western blotting protocol and buffers were the same as previously described (Watson and Tempel 2013) with a few changes. Whole cochlear samples were dissected on ice in Western lysis buffer by quickly removing both cochleae from the temporal bone. Lysis buffer was quickly perfused through the oval window, and cochleae were trimmed of as much excess bone, debris, vasculature, and neuronal tissue as possible while keeping the whole cochlea intact. In particular, the semi-circular canals were removed, and vestibular epithelia cleared leaving only tissue past the round and oval windows. Both cochleae from one mouse (constituting $n=1$) were pooled together into a sterile microtube containing 90 μ l lysis buffer and stored at -80 °C until further use. Cochleae were homogenized directly in the microtube with a new, sterile pestle (Argos Technologies), and protein concentrations of each sample were determined using a BCA assay (Pierce). Aliquots containing 10 μ g of each sample were mixed with sample buffer, denatured for 10 min at 50 °C, cooled, and then loaded directly onto a 4–20 % polyacrilamide RGEL (BioRad) or stored at -20 °C for later use. Primary antibody concentrations were 1:50,000 for β -actin (monoclonal mouse, Sigma) and 1:2,000 for PMCA2 (N-terminal, polyclonal rabbit, Affinity BioReagents). Secondary antibody concentrations were 1:70,000 (anti-mouse IgG from sheep, GE Life Sciences) and 1:2,000 (anti-rabbit IgG from donkey, GE Life Sciences), respectively. The ECL Prime kit (GE

Healthcare) was used to generate the chemiluminescent signal which was visualized immediately using a Fotodyne Luminary/FX instrument (Fotodyne Inc.). Two blots containing all individuals of both ages (P16 and 5 weeks, with half of each age on each blot) of a particular genotype were run in parallel, transferred in parallel, and incubated in the same antibody solutions to directly compare PMCA2 expression differences by age. This process was performed at least twice for each sample, so each data point reflects at minimum a duplicate for each sample. PMCA2 expression in each lane was normalized to β -actin expression. A 10- μ g cochlear standard was also loaded onto each gel to allow us to standardize replicates between blots and approximate the relative level of expression between genotypes. The images published in this manuscript have been inverted to look like a conventional blot, and have not been manipulated in any other way. Quantification was done on the longest exposure for each set of paired blots where none of the pixels were saturated.

RESULTS

Maturation of Auditory Sensitivity in Normal and PMCA2 Mutant Mice

To assess how PMCA2 contributes to the maturation of auditory sensitivity after the onset of hearing, ABR thresholds of wild-type and PMCA2 mutant mice were compared. Mice lacking PMCA2 are completely deaf so the haploinsufficient strain, $+/dfw^{i5}$ (Watson and Tempel 2013), was used to determine the effect of a reduction of PMCA2 expression on auditory development. Mice begin to hear around post-natal day 12 (P12) and have a mature endocochlear potential by P14 (Mikaelian and Ruben 1965). To ensure consistently detectable auditory brainstem responses, thresholds were assessed first at P16, followed by 3 (P20 or P21), 4, 5, 7, and 9 weeks of age (Fig. 1A). Statistical differences between $+/dfw^{i5}$ and wild-type controls are indicated in each panel, and threshold differences between notable ages within a group are described in Table 1. While wild-type thresholds improved at nearly all frequencies between P16 and 3 weeks, thresholds in $+/dfw^{i5}$ stayed the same (5.6, 8, and 11.3 kHz) or were noticeably worse (16 kHz and above) at this stage in auditory development. Thus, 3 weeks of age marks a critical time point in the development of auditory transduction in wild-type and $+/dfw^{i5}$ mice, with overall sensitivity improving in one group and worsening in the other. Variability of $+/dfw^{i5}$ thresholds (Fig. 1B) suggests that sensitivity changes rapidly around 3 weeks of age in the mid-frequencies. Wild-type thresholds were unchanged after 3 weeks of age at each frequency tested. In $+/dfw^{i5}$, thresholds at 5.6 kHz never matured from P16 into adulthood suggesting that reduced PMCA2 expression can nega-

FIG. 1. Auditory development in $+/dfw^{j5}$ and wild-type littermates. **A** ABR sensitivity improves in wild-type animals at all frequencies between P16 and 3 weeks and is mature at each frequency by about 4 weeks of age. In contrast, sensitivity at most frequencies worsens for $+/dfw^{j5}$ between P16 and 3 weeks of age, and recovers in a frequency-dependent manner through adolescence. Error bars represent standard deviation. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ in a Bonferroni post hoc comparison between $+/dfw^{j5}$ and littermates at each age following a two-way ANOVA. See Table 1 for statistical comparisons between ages within a genotype. **B** Variation in ABR thresholds of $+/dfw^{j5}$ during development. The relatively large standard deviations for thresholds of $+/dfw^{j5}$ at 11.3 and 16 kHz at 3 weeks of age suggest that sensitivity is changing rapidly at this stage of development in these mice. In a Bartlett's test of equal variance, age had a significant effect on variation at these frequencies (Bartlett's statistic = 25.47, $P = 0.0001$ at 11.3 kHz and Bartlett's statistic = 46.27, $P < 0.0001$ at 16 kHz). At 32 and 40 kHz, the large standard deviation in $+/dfw^{j5}$ throughout adolescence is reflective of an occasional one to two individuals with relatively intact sensitivity at these frequencies. A Bartlett's test indicates variation during this developmental stage is homogenous (no significant effect of age) at these frequencies.

tively impact low-frequency hearing. Thresholds recovered at 11.3 and 16 kHz to wild-type levels by 4 weeks of age and partially improved at 22.6 kHz, but not at 32 or 40 kHz. Loss (and subsequent recovery) of sensitivity seen at 3 weeks of age in mice heterozygous for another PMCA2 null allele, dfw^{2f} , demonstrates this is not a unique feature of $+/dfw^{j5}$ (Fig. 2). Therefore, the mature auditory phenotype of haploinsufficient PMCA2 mice relies on frequency-dependent recovery of sensitivity between 3 and 4 weeks of age.

Atp2b2 Regulation After the Onset of Hearing

Improvements in wild-type auditory thresholds at the same age when thresholds worsen in $+/dfw^{j5}$ suggest

there exists a minimal required level of PMCA2 expression for normal auditory development. To see if *Atp2b2* (the gene that encodes PMCA2) regulation changes during this critical period, we quantified total *Atp2b2* mRNA as well as specific expression of neuronal transcript types, α - and β -*Atp2b2* (Silverstein and Tempel 2006). Expression levels were quantified using whole cochlear RNA preps from individual mice at P16, P20, and 5 weeks of age (Fig. 3). These three time points should adequately capture a snapshot of expression levels before, during, and after the critical 3-week time point. The main cell types contributing to *Atp2b2* expression in the prep are auditory hair cells and spiral ganglion neurons. Spiral ganglia express both α - and β -*Atp2b2* transcript types while auditory

TABLE 1
Statistical differences of auditory thresholds during development for $+/dfw^{i5}$ and controls

	5.6 kHz	8 kHz	11.3 kHz	16 kHz	22.6 kHz	32 kHz	40 kHz
$+/+$							
P16 vs. 3 week	*** ($t_{14}=5.8$)	n.s. ($t_{14}=2.8$)	*** ($t_{14}=4.5$)	* ($t_{14}=3.3$)	** ($t_{14}=4.0$)	*** ($t_{14}=6.8$)	*** ($t_{14}=5.7$)
P16 vs. 5 week	*** ($t_{12}=6.5$)	** ($t_{12}=4.0$)	*** ($t_{12}=4.6$)	n.s. ($t_{12}=2.8$)	*** ($t_{12}=4.8$)	*** ($t_{12}=6.2$)	*** ($t_{12}=6.1$)
3 week vs. 5 week	n.s. ($t_{16}=1.0$)	n.s. ($t_{16}=1.4$)	n.s. ($t_{16}=0.33$)	n.s. ($t_{16}=0.38$)	n.s. ($t_{16}=1.1$)	n.s. ($t_{16}=0.43$)	n.s. ($t_{16}=0.74$)
$+/dfw^{i5}$							
P16 vs. 3 week	n.s. ($t_{18}=0.40$)	n.s. ($t_{18}=0.61$)	n.s. ($t_{18}=1.1$)	*** ($t_{18}=6.4$)	* ($t_{18}=3.5$)	n.s. ($t_{18}=2.1$)	n.s. ($t_{18}=2.2$)
P16 vs. 5 week	n.s. ($t_{17}=0.99$)	n.s. ($t_{17}=1.3$)	*** ($t_{17}=4.6$)	n.s. ($t_{17}=1.9$)	n.s. ($t_{17}=0.36$)	n.s. ($t_{17}=2.5$)	n.s. ($t_{17}=2.4$)
3 week vs. 5 week	n.s. ($t_{17}=0.60$)	n.s. ($t_{17}=0.71$)	*** ($t_{17}=5.7$)	*** ($t_{17}=8.1$)	* ($t_{17}=3.1$)	n.s. ($t_{17}=0.48$)	n.s. ($t_{17}=0.18$)

n.s. not significant

t values for each post hoc comparison are given in parenthesis

* $P<0.05$, ** $P<0.01$ and *** $P<0.001$ in a Bonferroni post hoc comparison following a one-way ANOVA at each frequency for each genotype

hair cells predominately express the α -Atp2b2 transcript (Silverstein and Tempel 2006).

As previously reported (Watson and Tempel 2013), $+/dfw^{i5}$ expression of each transcript type was approximately half (~60 %) of wild-type expression (overall effect of genotype in a two-way ANOVA, $P<0.0001$ for all transcript types, $F_{1,28}=169.0$ for total Atp2b2, $F_{1,28}=121.9$ for α -Atp2b2, and $F_{1,28}=163.3$ for β -Atp2b2). Total transcript expression in both groups trends upwards between P16 and 5 weeks of age (Fig. 3A) and is statistically increased in $+/dfw^{i5}$ (overall effect of age in a two-way ANOVA, $F_{2,28}=5.492$, $P=0.0097$ and $t_{12}=2.448$, $P<0.05$ in a Bonferroni post hoc comparison). The α -Atp2b2 transcript approximately doubled in both wild-type and $+/dfw^{i5}$ mice between P16 and 5 weeks, which was statistically higher than levels at both P16 and P20 for each group (overall effect of age in a two-way ANOVA, $F_{2,28}=74.29$, $P<0.0001$) (Fig. 3B). In contrast, expression of β -Atp2b2 did not change in the cochlea during this time frame (Fig. 3C). The increase in α -Atp2b2, but not β -Atp2b2, in the cochlea of both wild-type and $+/dfw^{i5}$ mice suggests that the α -Atp2b2 transcript type is particularly important for auditory transduction.

To account for transcriptional or compensatory differences in $+/dfw^{i5}$, α and β -Atp2b2 expression levels in each $+/dfw^{i5}$ individual were normalized to the wild-type expression level for that transcript type and then compared (Fig. 3D). Preferential usage of α -Atp2b2 over β -Atp2b2 was seen at 5 weeks of age ($t_8=4.501$, $P=0.0020$ in a paired, two-way t test), but not at P16 or P20, suggesting a compensatory change in Atp2b2 regulation occurs after 3 weeks of age in $+/dfw^{i5}$ mice.

To determine whether the increase in α -Atp2b2 expression is a global change or if it is unique to the cochlea, brain stem samples from each group and age were also assessed for total, α and β -Atp2b2 expression (Fig. 4). Unlike the cochlea, total, α and β -Atp2b2 levels in the brain stem were relatively stable over this time frame in both groups (Fig. 4A–C) with no overall effect of age in a two-way ANOVA for total and β -Atp2b2. There was an overall effect of age in a two-way ANOVA for α -Atp2b2 ($F_{2,34}=6.086$, $P=0.0055$) caused by a small increase in expression of wild-type transcript at P20 ($t_9=3.331$, $P<0.01$ between P16 and P20 and $t_{11}=2.773$, $P<0.05$ between P20 and 5 weeks in a Bonferroni post hoc comparison). As in Fig. 3D, compensatory changes in $+/dfw^{i5}$ were assessed by normalizing brain stem α -Atp2b2 and β -Atp2b2 expression for each $+/dfw^{i5}$ individual to wild-type expression for each transcript (Fig. 4D). In the brain stem tissue of $+/dfw^{i5}$ individuals, there was again a small, but significant, preference for α over β -Atp2b2 transcript usage at 5 weeks of age when normalized to wild-type expression ($t_8=2.750$, $P=0.025$ in a paired, two-way t test).

FIG. 2. Auditory development in $+/dfw^{2J}$ and wild-type littermates. Like in $+/dfw^{i5}$ (Fig. 1), ABR sensitivity at most frequencies worsens for $+/dfw^{2J}$ between P16 and 3 weeks of age, and recovers in a frequency-dependent manner through adolescence. Thus, loss of sensitivity at 3 weeks is not a unique feature of $+/dfw^{i5}$ and is common to other $+/dfw^{null}$ mutants. Thresholds improve in wild-type controls at all frequencies between P16 and 3 weeks of age. Error bars represent standard deviation. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ in a Bonferroni post hoc comparison between $+/dfw^{2J}$ and littermates at each age following a two-way ANOVA.

PMCA2 Expression Increases After the Onset of Hearing in Wild-Type Mice

To examine if increased α -Atp2b2 expression in the cochlea results in an increase in PMCA2, protein levels in whole cochlea of wild-type and $+/dfw^{i5}$ mice at P16 and 5 weeks of age were assessed via Western blotting (Fig. 5A). Wild-type PMCA2 expression increased in the cochlea between P16 and 5 weeks

(Fig. 5B, open circles, $t_{11}=5.519$, $P=0.0002$ in an unpaired, two-tailed t test). However, in $+/dfw^{i5}$, despite an increase in α -Atp2b2 transcript, cochlear PMCA2 expression did not increase above P16 levels (Fig. 5B, gray squares, $t_{11}=0.2850$, $P=0.7809$ in an unpaired, two-tailed t test). These data suggest that an increase in cochlear PMCA2 expression may contribute to the maturation of auditory sensitivity after P16 in normal auditory development. Likewise,

FIG. 3. Cochlear α -Atp2b2 is upregulated by 5 weeks of age. **A** Total Atp2b2 expression increases in cochlea of $+/dfw^{i5}$ mice between P16 and 5 weeks of age. **B–C** Levels of α -Atp2b2, but not β -Atp2b2, increase for both wild-type and heterozygous mice throughout auditory development in the cochlea. * $P<0.05$, ** $P<0.01$, and *** $P<0.001$ in a Bonferroni post hoc test following a two-way ANOVA (panels A–C). **D** Relative expression of α -Atp2b2 and β -

Atp2b2 is shown for individual $+/dfw^{i5}$ mice at each age in the cochlea. Expression levels for both transcripts were normalized to the average wild-type transcript expression at each age. Preferential upregulation of α -Atp2b2 is apparent in the cochlea of $+/dfw^{i5}$ at 5 weeks of age and is not seen at P16 or P20. *** $P=0.0020$ in a paired, two-way t test. Error bars represent SEM.

FIG. 4. Brain stem *Atp2b2* expression during auditory development. Expression does not change for total (A), α - (B) or β - (C) *Atp2b2* by 5 weeks of age in the brain stem of wild-type or *+/-dfwⁱ⁵* mice. Scale bars are matched for α - and β -*Atp2b2* to illustrate the relative abundance of each transcript in the brain stem. * $P < 0.05$ and ** $P < 0.01$ in a Bonferroni post hoc test following a two-way ANOVA. D Relative expression of α -*Atp2b2* and β -*Atp2b2* is shown for individual *+/-dfwⁱ⁵* mice at each age in brain stem. Expression levels for both transcripts were normalized to the average wild-type transcript expression at each age, and a slight preference in α -*Atp2b2* over β -*Atp2b2* usage is seen at 5 weeks (* $P = 0.025$ in a paired, two-way *t* test). Error bars represent SEM.

unchanged PMCA2 expression in *+/-dfwⁱ⁵* may contribute to the worsening of auditory sensitivity after P16 in these mice.

Noncoding RNAs may be Involved in *Atp2b2* Regulation

Our RNA data suggested that, in both groups of mice, regulation of α -*Atp2b2* seemed to be particularly important during the developmental stage between P16 and 5 weeks of age. In wild-type mice, the increase in α -*Atp2b2* transcript was more indicative of the change in PMCA2 expression than were total or β -*Atp2b2* transcript levels. In *+/-dfwⁱ⁵*, although PMCA2 expression did not increase in the cochlea, the preferential increase in α -*Atp2b2* transcript over β -*Atp2b2* suggests an attempt to compensate for a lack of PMCA2 expression by upregulation of α -*Atp2b2*. We asked whether nearby genomic elements might regulate α -*Atp2b2* during auditory development.

The *Atp2b2* gene is over 300 kb long with several large intronic regions. It has become increasingly apparent that intronic and intergenic regions of the genome can serve important regulatory and functional roles in mammalian organisms (Consortium EP 2012). We noticed that two uncharacterized noncoding RNA genes, *Gm15082* (lnc82) and *Gm15083* (lnc83), sit complementary to the 5' end of *Atp2b2* (Fig. 6A). Expression of these RNAs was confirmed in the brain stem and cochlea of 5-week-old wild-type mice (Fig. 6B, C). The lnc82 RNA is primarily expressed in the brain stem with minimal expression in the cochlea, and lnc83 RNA is primarily expressed in the cochlea with negligible expression in the brain

stem (Fig. 6B). Abundance relative to total *Atp2b2* expression is also shown for each lncRNA and tissue type (Fig. 6C).

Both lncRNA transcripts were then quantified from the same cDNAs of both wild-type and *+/-dfwⁱ⁵* mice used to measure *Atp2b2* expression at P16, P20, and 5 weeks of age (Fig. 7). Expression levels of lnc82 and lnc83 in the brain stem, as well as lnc82 in the cochlea, are stable over this time course. Conversely, lnc83 expression is markedly higher at P16 in the cochlea of both wild-type (CBA) and *+/-dfwⁱ⁵* mice. Levels decrease at P20 in each genotype, and are lowest at 5 weeks of age. We cannot help but notice that downregulation of lnc83 seems to coincide with the upregulation of α -*Atp2b2* (compared to Fig. 4B) and that *Gm15083* (the gene encoding lnc83) spans the transcriptional start site and proximal promoter for α -*Atp2b2* (see Fig. 6A). The lnc83 transcript is also highly expressed in the cochlea (compared to the brain stem) where α -*Atp2b2* is the predominant transcript type. Taken together, these results suggest that lnc83 RNA might be involved in regulating α -*Atp2b2* expression and tissue specificity. Further functional studies will be required to confirm these hypotheses.

PMCA4 Does Not Compensate for Loss of PMCA2 in *+/-dfwⁱ⁵*

While our data suggest that changes in *Atp2b2* and PMCA2 expression may contribute to the maturation of auditory sensitivity in wild-type and *+/-dfwⁱ⁵* mice, there are likely changes in expression levels of other genes between P16 and 5 weeks of age. We postulated

FIG. 5. PMCA2 expression increases by 5 weeks in wild-type, but not in $+/\text{dfw}^{i5}$. **A** Western blots of cochlear tissue from $+/\text{dfw}^{i5}$ and wild-type littermates at each age were assessed for PMCA2 expression (see “Methods”). Representative examples of blots from wild-type (top) and $+/\text{dfw}^{i5}$ (bottom) mice that were used to compare PMCA2 expression at P16 and 5 weeks of age within a genotype. On average, the four cochlear samples from individual P16 wild-type mice have reduced PMCA2 band intensity compared to three 5-week wild-type mice. Conversely, there is no noticeable difference between cochlear PMCA2 band intensity in three $+/\text{dfw}^{i5}$ mice at P16 and four $+/\text{dfw}^{i5}$ mice at 5 weeks of age. **B** PMCA2 expression was normalized to β -actin band intensity (arbitrary units) and quantified from sets of paired blots run with a standard to compare expression within pairs and estimate the relative expression between genotypes. The blots shown in panel “A” represent one blot each from a paired set of each genotype. There is approximately a 50 % increase in cochlear PMCA2 expression between P16 and 5 weeks in wild-type mice. Cochlear PMCA2 expression does not increase by 5 weeks in $+/\text{dfw}^{i5}$, which likely contributes to abnormal maturation of auditory sensitivity in these animals.

that, in $+/\text{dfw}^{i5}$, other genes expressed during early auditory development might remain at higher levels to compensate for the reduction in PMCA2 expression. Furthermore, such a gene might account for the recovery

of sensitivity after 3 weeks of age in these mice. Other calcium handling mechanisms, especially in neuronal tissue, may help compensate for the increased calcium load. The mRNA for another calcium ATPase, PMCA4, which is encoded by the *Atp2b4* gene, is expressed at its highest levels in the developing cochlea at P12 (Furuta et al. 1998) and is aberrantly expressed in the stereocilia of adult null-PMCA2 mice (Wood et al. 2004). For these reasons, we considered PMCA4 as a candidate for compensation in $+/\text{dfw}^{i5}$. Quantification of cochlear cDNAs show a reduction of *Atp2b4* expression in both wild-type and $+/\text{dfw}^{i5}$ mice by 5 weeks of age (overall effect of age in a two-way ANOVA, $F_{3,28}=53.91$, $P<0.0001$) (Fig. 8), indicating its expression continues to decrease even after the onset of hearing in mice. As there were no expression differences between wild-type and $+/\text{dfw}^{i5}$ groups, it appears that PMCA4 does not compensate for reduced PMCA2 function in adult $+/\text{dfw}^{i5}$ mice.

DISCUSSION

Here, we examine the effect of PMCA2 on auditory maturation and show that this critical hearing protein is also important for the proper development of auditory function. Recently, the appearance of PMCA2 in rat stereocilia bundles was characterized and found to be expressed first at the base in P0 animals and at the apex by P6 (Chen et al. 2012). This finding, paired with the appearance of cochlear *Atp2b2* mRNA before the onset of hearing (Furuta et al. 1998), suggests that PMCA2 expression is established before coordinated auditory transduction begins. Our findings demonstrate that PMCA2 expression continues to change even after the onset of hearing and that an increase in cochlear PMCA2 may be a component of the auditory maturation process. We also find that a reduction of PMCA2 alters the course of auditory maturation during the third post-natal week in mice.

Expression of *Atp2b2* and PMCA2 is Dynamic After the Onset of Hearing

We report a specific elevation in $\alpha\text{-Atp2b2}$ expression in the cochlea occurring after the onset of hearing in both wild-type and $+/\text{dfw}^{i5}$ mice. This increase in $\alpha\text{-Atp2b2}$ transcript correlates to an increase in cochlear PMCA2 expression by 5 weeks of age in wild-type mice, but does not appear to influence protein expression in $+/\text{dfw}^{i5}$ mice. While it is unclear why PMCA2 expression does not also increase in $+/\text{dfw}^{i5}$, there appears to be a compensatory change in the transcriptional regulation of the $\alpha\text{-Atp2b2}$ and $\beta\text{-Atp2b2}$ transcript types between P16 and 5 weeks of age in both cochlear and brain stem tissue of these mice. It is possible that a change in $\alpha\text{-Atp2b2}$ regulation in $+/\text{dfw}^{i5}$ contributes to the partial recovery of auditory sensitivity by 5 weeks of

A

B

C

FIG. 6. Two lncRNA genes sit complementary to *Atp2b2* and are expressed in the brain stem and cochlea of wild-type mice. **A** The schematic shows the location of the 5' region of α -*Atp2b2* (black exons) on the antisense strand of Chr. 6. The *Atp2b2* gene is transcribed from the reverse strand while two predicted long noncoding RNA genes (open or closed teal exons) sit complementary to the 5' region of *Atp2b2* on the forward strand of Chr. 6. *Gm15082* (or *Inc83*) spans the α -*Atp2b2* transcriptional start, and *Gm15082* (*Inc82*) sits between the second and third untranslated exons of the

α -*Atp2b2* transcript. The β -*Atp2b2* transcriptional start and translational start exons are also shown for reference. Exon sizes and relative positions on Chr. 6 are to scale. **B–C** lncRNA genes are expressed in a tissue-specific manner in wild-type mice with *Inc82* expression predominating in the brain stem and *Inc83* expression predominating in the cochlea. Graphs represent absolute expression levels (**B**) and abundance relative to total *Atp2b2* expression in wild-type mice (**C**). Error bars represent SEM.

FIG. 7. *Inc83* is downregulated in the cochlea by 5 weeks of age. Levels of *Inc82* and *Inc83* were quantified in the brain stem and cochlea of wild-type and $+/\text{dfw}^{j5}$ mice during auditory development. Expression of *Inc82* and *Inc83* in the brain stem, as well as *Inc82* in the cochlea, does not change over this time frame, and there are no grouped differences

between wild-type and heterozygous littermates. For both wild-type and $+/\text{dfw}^{j5}$, *Inc83* expression in the cochlea is significantly downregulated between P16, P20, and 5 weeks of age. *** $P < 0.001$ in a Bonferroni post hoc comparison following a two-way ANOVA. Error bars represent SEM.

FIG. 8. Developmental *Atp2b4* (PMCA4) expression in $+/dfw^{i5}$ mice. *Atp2b4* levels were quantified in the cochlea of $+/dfw^{i5}$ and controls to determine if PMCA4 compensates for PMCA2 deficiency in these mice. There is a significant overall effect of age ($F_{2,28}=53.91$, $P<0.0001$) indicating that PMCA4 expression decreases by 5 weeks, but no overall effect of genotype in these mice ($F_{1,28}=1.484$, $P=0.2347$) in a two-way ANOVA. *** $P<0.001$ relative to P16 and P20 in a Bonferroni post hoc comparison following a two-way ANOVA. Error bars represent SEM.

age in this strain, although it is also likely that compensatory changes in other calcium handling mechanisms in hair cell stereocilia (Lumpkin and Hudspeth 1998) may also occur. Unchanged PMCA2 expression in $+/dfw^{i5}$ cochleae, however, is consistent with the altered development of auditory sensitivity in these mice and suggests a role for PMCA2 in auditory maturation.

We also characterize the expression levels of two long noncoding RNA gene transcripts, *lnc82* and *lnc83*, that sit on the opposite strand of the 5' region of the *Atp2b2* gene. The *lnc83* expression is oppositely correlated with α -*Atp2b2* expression in the cochlea of both wild-type and $+/dfw^{i5}$ mice. The transcriptional start site of α -*Atp2b2* sits in the intronic region of the *lnc83* gene suggesting that *lnc83* might interfere specifically with α -*Atp2b2* transcription via indirect or direct mechanisms. Recently, examples of long noncoding RNAs acting as transcriptional repressors support this view (Huarte et al. 2010; Wang and Chang 2011; Maamar et al. 2013). The genomic proximity and expression patterns of the *Atp2b2* and *Gm15083* genes and their resulting transcripts implicate *lnc83* as a potential regulator of α -*Atp2b2* expression and tissue specificity. We expect that further studies on the mechanism of *lnc83* and other long noncoding RNAs in the mouse auditory system will reveal more detailed regulatory roles.

PMCA2 as a Component of Auditory Maturation

Consistent with other reports, auditory sensitivity of wild-type mice improves between P16 and 3 weeks of

age (Mikaelian and Ruben 1965; Shnerson and Pujol 1981; Song et al. 2006). However, two reduced PMCA2 expression strains, $+/dfw^{i5}$ and $+/dfw^{2J}$, demonstrate a distinct loss of auditory sensitivity at 3 weeks of age with frequency-dependent recovery by 5 weeks of age. Worsening of thresholds between P16 and 3 weeks may be attributed, in part, to the presence of PMCA1 mRNA in outer hair cells at P16 which disappears by P21 (Furuta et al. 1998). Compensation in hair cell stereocilia by PMCA4, as seen in adult dfw^{2J}/dfw^{2J} (Wood et al. 2004), is not predicted to contribute to frequency-dependent recovery of sensitivity in $+/dfw^{i5}$ since *Atp2b4* transcript is expressed at equivalent levels in the cochlea of these mice as in wild-type controls, suggesting that another mechanism for compensation occurs in these mice.

It is also possible that the amount of PMCA2 expressed in these strains at P16 is adequate for this stage in development, but that by 3 weeks of age, this level is no longer sufficient for normal auditory transduction. The third post-natal week is a critical period in auditory refinement and maturation of altricial mammals. Many features of both the central and peripheral auditory systems become “adult-like” during this time and include maturation of the endocochlear potential (Woolf et al. 1986; Sadanaga and Morimitsu 1995) changes in cochlear morphology and mechanics (Kraus and Aulbach-Kraus 1981; Shnerson et al. 1981; Mills and Rubel 1996; Overstreet et al. 2002; Emadi and Richter 2008; Song et al. 2008) as well as reorganization and refinement of tonotopicity (Echteler et al. 1989; Muller 1996; Yu et al. 2007).

A closer examination at each of the frequencies tested in Fig. 1 lends insight into the mechanism for PMCA2-related hearing loss in mice. At 5.6 and 8 kHz, ABR thresholds do not change dramatically after P16, neither worsening at 3 weeks nor improving thereafter. Why thresholds never improve at 5.6 kHz is a matter of speculation, but indicates that loss of sensitivity at these lowest frequencies in a PMCA2 mutant might be attributed to a different mechanism than hearing loss at higher frequencies in the same animals. Nonetheless, loss at 5.6 kHz suggests the necessity of PMCA2 for normal low-frequency hearing. That thresholds in $+/+$ and $+/dfw^{i5}$ at 8 kHz are virtually identical throughout development suggests this frequency is the most resistant to PMCA2 dysfunction and might represent the tonotopic location where there is a switch between low- and high-frequency mechanisms for hearing loss. Thresholds at 11.3 kHz are indistinguishable from controls at every age but the critical 3 week time point where maturation of thresholds lags by 1 week.

At frequencies at and above 16 kHz, the degree of hearing loss at 3 weeks of age is at least 35–

45 dB SPL. A 40–50 dB shift is characteristic of a lack in outer hair cell function (Ryan and McGee 1977; Prosen et al. 1978; Stebbins et al. 1979) and indicates that, at these frequencies, $+/\text{dfw}^{i5}$ does not provide enough PMCA2 activity at 3 weeks to fully support the signal amplification function attributed to outer hair cells (Brownell et al. 1985; Evans and Dallos 1993). At 16 and 22.6 kHz, $+/\text{dfw}^{i5}$ animals are able to fully or partially recover sensitivity throughout adolescence, respectively. These frequencies seem to represent the tonotopic location where transduction is sensitive, and yet the most receptive, to a reduction in PMCA2 expression and compensatory changes, respectively.

Finally, at 32 and 40 kHz, $+/\text{dfw}^{i5}$ mice maintain the 40–45 dB shift in sensitivity through 9 weeks of age. It is characteristic at these high frequencies to have a significant hearing loss due to a reduction in PMCA2 function (McCullough and Tempel 2004). Recent work suggests that high-frequency hair cells in PMCA2 mutants may be the most susceptible to calcium overload and subsequent death (Spiden et al. 2008; Bortolozzi et al. 2010; Watson and Tempel 2013) because they have an equivalent density of PMCA2 molecules as low-frequency hair cells (Chen et al. 2012) despite having larger MET channel conductances (Ricci et al. 2003; Beurg et al. 2006). These data support the idea that a mechanism involving Ca^{2+} homeostasis contributes to loss at these frequencies and that compensatory changes occurring after 3 weeks in $+/\text{dfw}^{i5}$ cannot augment function in these high-frequency hair cells.

The relatively dramatic differences in sensitivity across the tonotopic axis of the mouse cochlea during this developmental stage suggest that Ca^{2+} clearance (and therefore cytosolic Ca^{2+} concentration) is a critical determinant of hair cell function, with a concomitant narrow window for PMCA2 function. Furthermore, we propose that Ca^{2+} handling mechanisms continue to mature in the mouse cochlea well after the onset of hearing as evidenced by the dynamic changes in *Atp2b2* and PMCA2 expression between P16 and 5 weeks of age.

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Disclosure

The authors declare that they have no conflicts of interest, financial or otherwise, regarding this work.

REFERENCES

- BEURG M, EVANS MG, HACKNEY CM, FETTIPLACE R (2006) A large-conductance calcium-selective mechanotransducer channel in mammalian cochlear hair cells. *J Neurosci Off J Soc Neurosci* 26:10992–11000
- BORTOLOZZI M, BRINI M, PARKINSON N, CRISPINO G, SCIMEMI P, DE SIATI RD, DI LEVA F, PARKER A, ORTOLANO S, ARSLAN E, BROWN SD, CARAFOLI E, MAMMANO F (2010) The novel PMCA2 pump mutation Tommy impairs cytosolic calcium clearance in hair cells and links to deafness in mice. *J Biol Chem* 285:37693–37703
- BROWNELL WE, BADER CR, BERTRAND D, DE RIBAUPIERRE Y (1985) Evoked mechanical responses of isolated cochlear outer hair cells. *Science* 227:194–196
- CHEN Q, MAHENDRASINGAM S, TICKLE JA, HACKNEY CM, FURNESS DN, FETTIPLACE R (2012) The development, distribution and density of the plasma membrane calcium ATPase 2 calcium pump in rat cochlear hair cells. *Eur J Neurosci* 36:2302–2310
- CONSORTIUM EP ET AL (2012) An integrated encyclopedia of DNA elements in the human genome. *Nature* 489:57–74
- DUMONT RA, LINS U, FILOTEO AG, PENNISTON JT, KACHAR B, GILLESPIE PG (2001) Plasma membrane Ca^{2+} -ATPase isoform 2a is the PMCA of hair bundles. *J Neurosci Off J Soc Neurosci* 21:5066–5078
- ECHTELER SM, ARJMAND E, DALLOS P (1989) Developmental alterations in the frequency map of the mammalian cochlea. *Nature* 341:147–149
- EMADI G, RICHTER CP (2008) Developmental changes of mechanics measured in the gerbil cochlea. *J Assoc Res Otolaryngol* 9:22–32
- EVANS BN, DALLOS P (1993) Stereocilia displacement induced somatic motility of cochlear outer hair cells. *Proc Natl Acad Sci U S A* 90:8347–8351
- FURUTA H, LUO L, HEPLER K, RYAN AF (1998) Evidence for differential regulation of calcium by outer versus inner hair cells: plasma membrane Ca -ATPase gene expression. *Hear Res* 123:10–26
- HUARTE M, GUTTMAN M, FELDSEER D, GARBER M, KOZIOL MJ, KENZELMANN-BROZ D, KHALIL AM, ZUK O, AMIT I, RABANI M, ATTARDI LD, REGEV A, LANDER ES, JACKS T, RINN JL (2010) A large intergenic noncoding RNA induced by p53 mediates global gene repression in the p53 response. *Cell* 142:409–419
- KELLEY MW (2006) Regulation of cell fate in the sensory epithelia of the inner ear. *Nat Rev Neurosci* 7:837–849
- KOZEL PJ, FRIEDMAN RA, ERWAY LC, YAMOA EN, LIU LH, RIDDLE T, DUFFY JJ, DOETSCHMAN T, MILLER ML, CARDELL EL, SHULL GE (1998) Balance and hearing deficits in mice with a null mutation in the gene encoding plasma membrane Ca^{2+} -ATPase isoform 2. *J Biol Chem* 273:18693–18696
- KRAUS HJ, AULBACH-KRAUS K (1981) Morphological changes in the cochlea of the mouse after the onset of hearing. *Hear Res* 4:89–102
- LUMPKIN EA, HUDSPETH AJ (1998) Regulation of free Ca^{2+} concentration in hair-cell stereocilia. *J Neurosci Off J Soc Neurosci* 18:6300–6318
- MAAMAR H, CABILI MN, RINN J, RAJ A (2013) linc-HOXA1 is a noncoding RNA that represses Hoxa1 transcription in cis. *Gene Dev* 27:1260–1271
- MCCULLOUGH BJ, TEMPEL BL (2004) Haplo-insufficiency revealed in deafwaddler mice when tested for hearing loss and ataxia. *Hear Res* 195:90–102
- MIKAELIAN D, RUBEN RJ (1965) Development of hearing in the normal Cba-J mouse: correlation of physiological observations

- with behavioral responses and with cochlear anatomy. *Acta Otolaryngol* 59:451–461
- MILLS DM, RUBEL EW (1996) Development of the cochlear amplifier. *J Acoust Soc Am* 100:428–441
- MULLER M (1996) The cochlear place-frequency map of the adult and developing Mongolian gerbil. *Hear Res* 94:148–156
- OVERSTREET EH, TEMCHIN AN, RUGGERO MA (2002) Passive basilar membrane vibrations in gerbil neonates: mechanical bases of cochlear maturation. *J Physiol* 545:279–288
- PROSEN CA, PETERSEN MR, MOODY DB, STEBBINS WC, HAWKINS JE JR (1978) Permanent threshold shift and cochlear hair cell loss in the kanamycin-treated guinea pig. *Otolaryngology* 86:ORL-886–ORL-887
- RICCI AJ, CRAWFORD AC, FETTIPLACE R (2003) Tonotopic variation in the conductance of the hair cell mechanotransducer channel. *Neuron* 40:983–990
- RYAN A, MCGEE TJ (1977) Development of hearing loss in kanamycin treated chinchillas. *Ann Otol Rhinol Laryngol* 86:176–182
- SADANAGA M, MORIMITSU T (1995) Development of endocochlear potential and its negative component in mouse cochlea. *Hear Res* 89:155–161
- SHNERSON A, PUJOL R (1981) Age-related changes in the C57BL/6J mouse cochlea. I. Physiological findings. *Brain Res* 254:65–75
- SHNERSON A, DEVIGNE C, PUJOL R (1981) Age-related changes in the C57BL/6J mouse cochlea. II. Ultrastructural findings. *Brain Res* 254:77–88
- SILVERSTEIN RS, TEMPEL BL (2006) Atp2b2, encoding plasma membrane Ca²⁺-ATPase type 2, (PMCA2) exhibits tissue-specific first exon usage in hair cells, neurons, and mammary glands of mice. *Neuroscience* 141:245–257
- SONG L, MCGEE J, WALSH EJ (2006) Frequency- and level-dependent changes in auditory brainstem responses (ABRs) in developing mice. *J Acoust Soc Am* 119:2242
- SONG L, MCGEE J, WALSH EJ (2008) Development of cochlear amplification, frequency tuning, and two-tone suppression in the mouse. *J Neurophys* 99:344–355
- SPIDEN SL, BORTOLOZZI M, DI LEVA F, DE ANGELIS MH, FUCHS H, LIM D, ORTOLANO S, INGHAM NJ, BRINI M, CARAFOLI E, MAMMANO F, STEEL KP (2008) The novel mouse mutation oblivion inactivates the PMCA2 pump and causes progressive hearing loss. *PLoS Genet* 4:e1000238
- STEBBINS WC, HAWKINS JE JR, JOHNSON LG, MOODY DB (1979) Hearing thresholds with outer and inner hair cell loss. *Am J Otolaryngol* 1:15–27
- STREET VA, MCKEE-JOHNSON JW, FONSECA RC, TEMPEL BL, NOBEN-TRAUTH K (1998) Mutations in a plasma membrane Ca²⁺-ATPase gene cause deafness in deafwaddler mice. *Nat Genet* 19:390–394
- WANG KC, CHANG HY (2011) Molecular mechanisms of long noncoding RNAs. *Mol Cell* 43:904–914
- WATSON CJ, TEMPEL BL (2013) A new Atp2b2 deafwaddler allele, dfw (i5), interacts strongly with Cdh23 and other auditory modifiers. *Hear Res* 304:41–48
- WOOD JD, MUCHINSKY SJ, FILOTEO AG, PENNISTON JT, TEMPEL BL (2004) Low endolymph calcium concentrations in deafwaddler2J mice suggest that PMCA2 contributes to endolymph calcium maintenance. *J Assoc Res Otolaryngol* 5:99–110
- WOOLF NK, RYAN AF, HARRIS JP (1986) Development of mammalian endocochlear potential: normal ontogeny and effects of anoxia. *Am J Physiol* 250:R493–R498
- YAMOAH EN, LUMPKIN EA, DUMONT RA, SMITH PJ, HUDSPETH AJ, GILLESPIE PG (1998) Plasma membrane Ca²⁺-ATPase extrudes Ca²⁺ from hair cell stereocilia. *J Neurosci Off J Soc Neurosci* 18:610–624
- YU X, SANES DH, ARISTIZABAL O, WADGHIRI YZ, TURNBULL DH (2007) Large-scale reorganization of the tonotopic map in mouse auditory midbrain revealed by MRI. *Proc Natl Acad Sci U S A* 104:12193–12198