Tonotopic Gradient in the Developmental Acquisition of Sensory Transduction in Outer Hair Cells of the Mouse Cochlea

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Lelli A, Asai Y, Forge A, Holt JR, Géleóc GS. Tonotopic gradient in the developmental acquisition of sensory transduction in outer hair cells of the mouse cochlea. J Neurophysiol 101: 2961–2973, 2009. First published April 1, 2009; doi:10.1152/jn.00136.2009. Inner ear hair cells are exquisite mechanosensors that transduce nanometer scale deflections of their sensory hair bundles into electrical signals. Several essential elements must be precisely assembled during development to confer the unique structure and function of the mechanotransduction apparatus. Here we investigated the functional development of the transduction complex in outer hair cells along the length of mouse cochlea acutely excised between embryonic day 17 (E17) and postnatal day 8 (P8). We charted development of the stereociliary bundle using scanning electron microscopy; FM1-43 uptake, which permeates hair cell transduction channels, mechanotransduction currents evoked by rapid hair bundle deflections, and mRNA expression of possible components of the transduction complex. We demonstrated that uptake of FM1-43 first occurred in the basal portion of the cochlea at P0 and progressed toward the apex over the subsequent week. Electrophysiological recordings obtained from 234 outer hair cells between E17 and P8 from four cochlear regions revealed a correlation between the pattern of FM1-43 uptake and the acquisition of mechanotransduction. We found a spatiotemporal gradient in the properties of transduction including onset, amplitude, operating range, time course, and extent of adaptation. We used quantitative RT–PCR to examine relative mRNA expression of several hair cell myosins and candidate tip-link molecules. We found spatiotemporal expression patterns for mRNA that encodes cadherin 23, protocadherin 15, myosins 3a, 7a, 15a, and PMCA2 that preceded the acquisition of transduction. The spatiotemporal expression patterns of myosin 1c and PMCA2 mRNA were correlated with developmental changes in several properties of mechanotransduction.

INTRODUCTION

Inner ear hair cells convert mechanical information into electrical signals that are transmitted to the brain. Although the biophysical properties of hair cell mechanotransduction have been well characterized in bullfrogs (Assad and Corey 1992; Corey and Hudspeth 1979, 1983; Eatoek et al. 1987), turtles (Crawford et al. 1989, 1991; Ricci and Fettiplace 1998; Wu et al. 1999), and rats (Beurg et al. 2006; Kennedy et al. 2003), the genetic basis of hair cell transduction remains elusive. To facilitate molecular identification of components of the hair cell mechanotransduction cascade, we have opted to focus on the hair cells of the mouse inner ear, a model system that is physiologically challenging but genetically accessible. Furthermore, because there are >65 mouse models of human deafness, many of which have a congenital onset, we were interested to examine the functional development of mouse auditory hair cells with particular attention devoted to the development and maturation of sensory transduction.

Developmental acquisition of mechanosensitivity has been described previously in vestibular hair cells of the mouse (Géleóc and Holt 2003) and in auditory hair cells of the chicken (Si et al. 2003) and rat (Kennedy et al. 2003; Waguespack et al. 2007). We were interested to extend these observations and investigate whether development of mechanotransduction in mouse auditory hair cells more closely resembled that of mouse vestibular hair cells or development of mechanotransduction in auditory hair cells of other species. Interestingly, Géleóc and Holt (2003) described a rapid acquisition of mechanotransduction, within ~18 h, in mouse vestibular hair cells between embryonic day (E) 16 and E17, whereas in auditory hair cells Waguespack et al. (2007) and Si et al. (2003) found a more gradual trend that extended from several days to over a week, respectively. Furthermore, both studies on auditory hair cells reported a gradual maturation in several of the biophysical properties of mechanotransduction including the maximal current amplitudes, the range of bundle deflections over which the currents were activated and the time course of adaptation. This also differed from the development of mechanotransduction in mouse vestibular hair cells, which had all the qualities of mature transduction at the onset at E17. Based on that observation, Géleóc and Holt (2003) proposed a developmental model in which the elements of the transduction apparatus are assembled in the cell body and rapidly transported to the tips of stereocilia where they are inserted and become functional with all the qualitative properties of mature transduction. Waguespack et al. (2007) proposed an alternate model in which the elements of the transduction apparatus are transported to the tips of stereocilia separately and are inserted in a “step-wise” fashion giving rise to an incremental acquisition of the properties of mechanotransduction.

Here we characterize the developmental properties of transduction in hair cells of the mouse auditory organ between E17 and the end of the first postnatal week. We examined the spatiotemporal pattern of FM1-43 uptake, a fluorescent dye that permeates transduction channels. Next we examined the onset of transduction electrophysiologically which allowed us to quantify several biophysical properties including current amplitude, operating range, and the time course and extent of adaptation. For the electrophysiological study, we focused on outer hair cells because they are more abundant, more accessible, and their bundles are more easily stimulated. We found a discrete onset such that for a given region the cells went from 10 to 90% functional within ~21 h. Cells first became func-
tional in the base at P0 followed by a wave of acquisition that progressed toward the apex by P2. A gradual maturation in the properties of transduction followed over the subsequent few days.

We also used quantitative RT-PCR (qPCR) to examine the spatiotemporal pattern of mRNA expression of several genes the protein products of which have been localized to hair bundles and have a putative role in mechanotransduction. While hair bundle localization of the protein product is required to invoke a role in bundle development and/or mechanotransduction, we opted to use a qPCR approach because it is an extremely sensitive assay able to detect low abundance transcripts and because it allows for quantitative comparison between samples, two advantages that are more problematic for an immunolocalization approach. We found that myosin 1c mRNA expression was correlated with the onset of transduction, consistent with the suggestion that the protein may be involved in the developmental assembly of the transduction apparatus (Géléc and Holt 2003). Expression of mRNA that encodes the calcium pump PMCA2 was correlated with refinement of several mechanotransduction properties, consistent with the hypothesis that PMCA2 activity can regulate calcium-dependent functions of hair cell transduction (Si et al. 2003).

**METHODS**

**Tissue preparation**

Protocols approved by the Animal Care Committee of the University of Virginia (Protocol No. 3123) were used to harvest the organ of Corti from Swiss Webster mice (Hilltop Lab Animals, Scottsdale, PA, and Taconic Farms, Germantown, NY) from embryonic day 17 (E17) to postnatal day 8 (P8). E1 refers to the first day of gestation (plug day) in timed pregnant females placed overnight with a male. P0 refers to the day of birth, typically around E20 for this strain. Embryos were removed by Cesarean section around midday and killed by rapid decapitation. Postnatal mouse pups were killed by rapid decapitation. The temporal bones were excised and bathed in MEM (In-vitrogen, Carlsbad, CA) supplemented with 10 mM HEPES (pH 7.4). The organ of Corti was gently dissected away from its accessory structures and the tectorial membrane was impelled without the use of enzyme. Depending on the goal of the experiment, the organ was either maintained intact (imaging and scanning electron microscopy) or divided into quarter turns (physiology and quantitative PCR): basal quarters were determined in real time by using Zeiss Image Browser software (Fig. 2A). The four quarters were determined in real time by using Zeiss Image Browser to measure the entire ~1.5-turn length of the organ of Corti. Because the organ elongates as a function of development, from ~4 mm at P0 to ~6 mm at P8, we used this method to divide the tissue into four equal sections at each developmental stage. For electrophysiology, the excised turns were mounted on round glass coverslips. A pair of thin glass fibers previously glued to the coverslip was placed on the edge of the tissue to stabilize it in a flat position. For imaging, the tissues were placed on a round coverslip previously coated with Cell-Tak (BD Bioscience, Franklin Lakes, NJ).

**Confocal imaging**

The styryl dye FM1-43 (Invitrogen) was applied to the mounted tissue at 5 μM for 10 s. The dye that partitioned into the outer leaflet of the cell membrane was washed out during three full bath replace-ments. Outer hair cells were imaged 5 min after exposure to FM1-43 using an LSM 510 confocal microscope (Zeiss) equipped with ×5 and ×40 dry objectives and a pinhole adjusted to one airy unit. When imaging the entire organ, tissues observed under ×5 and z-series acquisition were performed (focal interval: 8.3 μm). Images were acquired as eight-bit images using identical gain, contrast, pinhole and laser intensity settings. Two-dimensional projections of 10 image planes of the organ of Corti were generated using Zeiss LSM Image Browser and fluorescence intensity profiles were derived from analysis with Metamorph 6.2 (Molecular Devices, Sunnyvale, CA).

**Scanning electron microscopy (SEM)**

The auditory bullae of mice aged P0 and P6 were isolated and the cochleae exposed. In each cochlea, part of the cartilaginous cochlear wall was removed. Fixative solution was gently perfused into the inner ear through this opening. The entire bulla was then immersed in fixative, and fixation continued for a total of 2 h at room temperature while the sample was slowly rotated. The primary fixative was 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.35, with 3 mM CaCl₂. The cochleae were postfixed in 1% OsO₄ in the same buffer. After this, under 0.1 M cacodylate buffer, the organ of Corti was isolated from the cochlea and the tectorial membrane was gently pulled away. The tissue was then double processed with thiocarbohydrazide followed by OsO₄ (Davies and Forge 1987) before dehydration through an alcohol series and critical point drying with CO₂. After mounting on specimen support stubs, the samples were sputter coated with platinum. Samples were examined with a JEOL JSM 6700F cold field emission scanning electron microscope operating at 3 or 5 kV. Images of the hair bundles of outer hair cells of all three rows were collected from the apical, middle, and basal regions of the organ of Corti spiral. At each location, the hair bundles were viewed both from behind the longest row of stereocilia to view the height of the hair bundle as well as approximately perpendicular to the apical surface of the hair cell or toward the inner aspect of the bundle to examine its overall morphology and composition. To estimate bundle height, measurements were made from stereopairs of images at calibrated magnifications of ×20,000 or occasionally ×10,000. Measurements were taken from bundles viewed from the lateral side toward the medial side (i.e., from the stria toward the modiolus), so that the row of longest stereocilia were imaged from the “rear.” Samples were tilted and rotated so that the row of longest stereocilia were approximately perpendicular to the direction of view. Stereomaging was used to apply corrections for parallax errors. Measurements were made from three to five (occasionally 6) stereocilia closest to the kinocilium on each of at least three different bundles at each location in three different cochlea at each age.

**Electrophysiological recording**

Recordings were performed in standard artificial perilymph solution containing (in mM): 144 NaCl, 0.7 NaH₂PO₄, 5.8 KCl, 1.3 CaCl₂, 0.9 MgCl₂, 5.6 d-glucose, and 10 HEPES–NaOH, adjusted to pH 7.4 and 320 mosmol/kg. Vitamins (1:50) and amino acids (1:100) were added from concentrates (Invitrogen). Hair cells were viewed from the apical surface using an upright Axiostar FS microscope (Zeiss, Oberkochen, Germany) equipped with a ×63 water-immersion objective with differential interference contrast optics. Recording pipettes (2–5 MΩ) were pulled from borosilicate capillary glass (Garner Glass, Claremont, CA) and filled with intracellular solution containing (in mM): 135 KCl, 5 EGTA-KOH, 10 HEPES, 2.5 K₂ATP, 3.5 MgCl₂, 0.1 CaCl₂, pH 7.4. Currents were recorded under whole cell voltage-clamp at room temperature using an Axopatch Multiclamp 700A (Molecular devices, Palo Alto, CA), filtered at 10 kHz with a low-pass Bessel filter, digitized at ≥20 kHz with a 12-bit acquisition board (Digidata 1322) and pClamp 8.2 (Molecular Devices, Palo Alto, CA) and stored on disk for off-line analysis using OriginPro 7.1 (MicroCal).
Software, Northampton, MA). Results are presented as means ± SE (unless otherwise stated).

**Mechanical stimulation**

Mechanical stimuli were transmitted via a stiff glass probe mounted on a one-dimensional PICMA chip piezo actuator (Physik Instruments, Waldbronn, Germany) driven by a 400-mA ENV-400 Amplifier (Piezosystem Jena) (Stauffer and Holt 2007). The tip of the probe was fire polished (Fire polisher, H602, World Precision Instruments, Sarasota, FL) to ∼3–5 μm in diameter to fit the V-shaped stereociliary bundle (Stauffer and Holt 2007). Deflections were evoked by applying voltage steps low-pass filtered with an 8-pole Bessel filter (Khron-Hite, Brockton, MA) at 50 kHz to eliminate residual pipette resonance. Hair bundle deflections were monitored using a C2400 CCD camera (Hamamatsu). Voltage steps were used to calibrate the motion of the stimulus probe ±2 μm relative to its rest position. Video images of the probe were recorded to confirm absence of off-axis motion and calibrate the probe motion (spatial resolution of ∼4 nm).

The 10–90% rise time of the probe was ∼20 μs.

The position of the pipette and bundle were continuously monitored before, during, and after each recording using video microscopy to ensure that the stimulus pipette and the hair bundle moved in unison. Although differential movement at sub video frame rates could not be ruled out, the continuous current records and the consistent frame-to-frame positions of the stimulus pipettes and hair bundles that were clear at video rates, suggested that this was unlikely.

**Quantitative PCR**

cDNA banks for quantitative PCR were prepared from mouse cochleae, which included both sensory and nonsensory cells collected at seven developmental stages: E17, E18, P0, P2, P4, P6, and P8; divided into four quarters (see Tissue preparation). A total of 6–20 ears were dissected at each stage. To minimize RNA degradation, tissues were rapidly collected. The spiral ganglion was trimmed away, and the stria vascularis, Reissner’s membrane, and tectorial membrane were dissected at each stage. To minimize RNA degradation, tissues were rapidly collected. The spiral ganglion was trimmed away, and the stria vascularis, Reissner’s membrane, and tectorial membrane were dissected at each stage. Total RNA was measured with a spectrophotometer (Nanodrop, Santa Clara, CA). Electropherograms that show ribosomal RNAs S18 and S29 peaks as well as RIN values and RNA concentration were generated for each sample. Only samples with RIN values >8.0 were used for quantitative RT-PCR experiments.

One hundred nanogram of total RNA from each quarter was reverse transcribed with an iScript kit (Biorad, Hercules, CA) and used for single-color real time PCR with SYBR GreenER qPCR reagent (Invitrogen) and iCycler (Biorad) to test the expression level of β actin (Actb). To screen for contamination from genomic DNA, a no-reverse transcription control (same preparation but with water replacing the reverse transcription enzyme) was simultaneously tested. Once it was confirmed that the β actin amplicon was present in the transcribed sample but not in the no-RT control, 1 μg total RNA was used for linear amplification using RiboAmp RNA amplification kit (Arcturus, Mountain View, CA). The average yield of amplified RNA between quarters was 7.11 ± 0.75 μg/μl (n = 28). The amplified RNA from each quarter was reverse transcribed for the cDNA bank as described in the preceding text. Here we use standard convention with normal font to refer to the protein product of the gene/mRNA expression, which is shown in italics. Myosin 7a (Myo7a), myosin 1c (Myo1c), myosin 15a (Myo15a), myosin 3a (Myo3a), cadherin 23 (Cdh23), protocadherin 15 (Pcdh15), PMCA2 (Apb2h), and prestin (Slc26a5) were analyzed simultaneously in triplicate for each quarter and each developmental stage. Primer sets were designed using the PrimerQuest Software from Integrated DNA Technologies (San Diego, CA). Primers with melting temperatures ranging from 56.4 to 61°C produced amplicons of 83–122 bp (see Supplemental Table S1). Quantitative PCR reactions were designed according to the manufacturer’s instruction (SYBR GreenER qPCR reagent, Invitrogen). Each reaction (25 μl) included primers at 200 nM and cDNA generated from 12.5 ng amplified RNA. Amplicons were obtained over 40 cycles, and their purity was confirmed using the melting curve analysis. A dilution series of cDNA (1:1, 1:10, and 1:100) was used to validate the efficiency of each primer set. The cycle threshold (Ct) values of triplicates for each sample were averaged and normalized relative to the averaged Ct value of β actin (Fig. 7A) obtained from the same sample. The fold difference in mRNA expression between each sample was calculated following the comparative delta Ct method. For each gene of interest the data were normalized to most immature quarter, E17 apex, with the exception of Pcdh15 which was normalized against the most mature stage (P8, base) to illustrate the decrease in expression that occurred during development for that gene.

**Results**

**Development of the sensory hair bundle**

To screen for morphological changes that coincided with the onset of mechanotransduction, we used scanning electron microscopy to image hair bundles from mouse outer hair cells at birth (P0) and near the end of the first postnatal week (P6). For each stage, we divided the cochlea into three regions: base, middle and apex. At each stage and location, the hair bundles from outer hair cells were viewed from behind the tallest row of stereocilia, as well as from above, approximately perpendicular to the apical surface. We observed several developmental changes in hair bundle morphology that occurred throughout the first postnatal week and that differed from base to apex. As shown in Fig. 1, we observed that the tallest row of stereocilia in basal hair bundles were more uniform in height than tallest row stereocilia from middle and apical hair bundles, which had stereocilia that were taller in the center than in the lateral aspects. By P6, the difference among tallest row stereocilia had diminished in middle and apical hair bundles such that they more closely resembled P0 basal hair bundles. We also noticed that at P6 there was an increase in bundle height in apical hair bundles and surprisingly, a decrease in bundle height in outer hair cells from the base. Bundle height measurements were made from stereopairs of images in the base and apex at P0 and P6 (see Methods). Measurements were taken from bundles viewed from the lateral side so that the row of tallest stereocilia were imaged from behind. Samples were tilted and rotated so that the tallest stereocilia were approximately perpendicular to the direction of view. Measurements were taken from three to six stereocilia next to the kinocilium. We noted a significant difference in bundle height along the cochlea and during development (Fig. 1B). At birth, bundles were taller in the base than in the apex: 2.05 ± 0.01 versus 1.76 ± 0.01 μm, respectively. Interestingly, over the subsequent week, bundle height decreased significantly in the base (∼58%; P < 0.0001)

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1 The online version of this article contains supplemental data.
while it increased slightly in the apex (~10%; \( P < 0.0001 \)). At P6 bundles were 0.85 ± 0.02 \( \mu \)m in the base versus 1.95 ± 0.01 \( \mu \)m in the apex. A gradient in bundle height along the cochlea has been reported previously, but this is the first report to document that the gradient is established by bundle shortening at the basal end and lengthening at the apical end. The mechanisms that regulate the developmental changes in bundle height were not investigated. Less striking changes occurred in hair bundle height in the mid sections of the organ. Occasionally small immature bundles were observed at later stages but were not included in our analysis.

At P0 the staircase arrangement of stereocilia rows that typifies mature hair bundles was apparent throughout the cochlea, as demonstrated previously (Belyantseva et al. 2005). The staircase array became increasingly prominent at later stages (not shown) consistent with a report from Goodyear et al. (2005). Kinocilia, identified by their greater thickness and position behind the tallest row of stereocilia, were present along the organ of Corti at P0 but were absent by P6.

In mouse vestibular hair cells, we showed that the onset of transduction was concomitant with the appearance of tip links (Géléoc and Holt 2003). In this preparation, we found it difficult to discern tip links from the numerous other links present in developing bundles as reported elsewhere (reviewed by Nayak et al. 2007). As such, we did not chart the appearance of tip-links in our samples but note that Goodyear et al. (2005) observed oblique links as early as E17.5 in apical outer hair cell bundles that were in the right place to be tip links.

**FM1-43 uptake along the developing cochlea**

Because morphological development of outer hair cell bundles was evident throughout the first postnatal week, we were interested to identify when during development hair bundles became functional. As an initial assay, we used the styryl dye FM1-43, which permeates transduction channels (Gale et al. 2001; Géléoc and Holt 2003; Meyers et al. 2003). We quantified FM1-43 uptake in developing cochlear hair cells and found a precise spatiotemporal gradient in the acquisition of mechanotransduction channels. We used brief dye application (10 s) followed by a 5-min wash to assay for the presence of transduction channels open at rest. To confirm that our protocol selectively labeled transducing hair cells, we exposed the cells to 1 mM amiloride, an established transduction channel blocker, and then applied FM1-43. The fluorescent signal was significantly reduced, suggesting it was primarily the result of FM1-43 entry via amiloride-sensitive transduction channels, consistent with previous reports (Gale et al. 2001). The FM1-43 assay allowed us to examine dye uptake in all the hair cells along the entire cochlea at each developmental time point. The disadvantage of the approach is that it cannot reveal the biophysical status of the transduction machinery, which requires measurement of transduction current in a subset of hair cells, one cell at a time (see the next section).

We imaged whole-mount organs of Corti on a confocal microscope at 24-h intervals from P0 to P8. Figure 2A illustrates a bright-field view of the intact, whole-mount organ of Corti preparation. To minimize litter-to-litter variability in the developmental continuum, we used inbred pups from the same litter (from litters of \( \approx 10 \) pups) which allowed us to examine tissue harvested from pups with nearly identical genotypes, times of conception, and exposure to nearly identical environmental conditions. Live organs of Corti were prepared, bathed in FM1-43, mounted, and imaged 5 min after dye exposure (Fig. 2, B–E). We observed low level fluorescence in hair cells at the very basal portion of the cochlea as early as P0 (Fig. 2B). After 24 h, the uptake was more prominent in the basal portion and by P2 the FM1-43 signal was observed in the basal half of the organ (Fig. 2C). FM1-43 uptake progressed from the base toward the apex over the next few days. By P6, strong FM1-43 fluorescence was observed in \( \approx 90\% \) of the organ of Corti (Fig. 2E). FM1-43 uptake extended to all hair cells throughout the entire organ by P7 (not shown). To examine whether the onset of FM1-43 uptake began prior to birth, we also performed the experiment on tissue excised from E17 and E18 embryos. No signal was detected before birth even in the most basal portion of the cochlea. The entire experiment was repeated three times with three different litters, and we noted that the spatiotemporal pattern of FM1-43 uptake was consistent in all samples obtained from other litters at the various developmental stages.

To quantify the relative fluorescence intensity, we analyzed the images shown in Fig. 2, B–E, using a linescan drawn along the length of the organ of Corti. The line was 20 pixels wide, paralleled the curvature of the flat-mounted cochlea and included within its borders, fluorescence from both inner and outer hair cells. We plotted the average pixel intensity along the cochlea as a function of the linear distance from base to apex. The graph illustrates an increase in fluorescence intensity which expanded from the base toward the apex as function of
development between P0 and P6. As such, our quantitative data show a developmental gradient in the uptake of FM1-43 in mouse cochlear hair cells that is similar to a previous qualitative observation reported by Gale et al. (2001).

Acquisition of mechanosensitivity along the developing cochlea

Based on the spatiotemporal pattern of FM1-43 uptake shown in Fig. 2, we hypothesized that the onset of hair cell mechanosensitivity would follow a similar spatiotemporal pattern beginning at the base at P0 and extending to the apex by P7. However, because the FM1-43 can only assay for the acquisition of channels open at rest, we sought to examine the developmental onset of hair cell mechanosensitivity directly by deflecting hair bundles and measuring the response using the whole cell, tight-seal technique. Responses to mechanical stimulation were measured in hair cells of the developing cochlea from the four quarters shown in Fig. 2A: the most basal quarter referred to as BA (or base), the second quarter referred to as MB (or mid basal), the third quarter referred to as MA (or mid apical) and the most apical quarter referred to as AP (or apex). Responses to mechanical stimulation were measured in a total of 234 outer hair cells at stages that ranged from E17 to P8. The cells were bathed in artificial perilymph that contained 1.3 mM external calcium, and all recordings were made in voltage-clamp mode at a holding potential of $-64$ mV. Representative families of currents recorded from 32 individual hair cells are illustrated in Fig. 3. In contrast to the acquisition of transduction in vestibular hair cells (Géléc and Holt 2003), we were unable to evoke transduction currents from any of the hair cells examined prior to birth regardless of stimulus time course, amplitude, or direction and visual confirmation of substantial bundle deflections. Recordings from those cells revealed robust voltage-dependent currents that raised confidence that cells $<$P0 were viable but had not yet acquired mechanosensitivity. The earliest transduction currents that we observed were in response to step stimuli oriented along the bundle’s morphological axis of symmetry in outer hair cells from the base and apex.
mid basal quarter at P0 (Fig. 3). Interestingly, at this stage we were able to evoke transduction currents in 83% (10/12) of the cells in the base and also 83% (10/12) in the mid basal quarter (Fig. 4A and Supplemental Table S2). The transduction currents recorded at P0 appeared mature in several respects with average peak amplitudes of $-257.5 \pm 36.7$ pA ($n=10$) at the base and $-195.1 \pm 40.3$ pA ($n=10$) in the mid basal quarter. We were unable to evoke transduction currents in outer hair cells of the mid apical quarter or apex. By P1, small transduction currents ($-118.8 \pm 34.6$ pA, $n=5$) could be elicited from 56% of the cells (5/9) in the mid apical quarter, whereas none were detected in apical cells (Fig. 4). However, by P2, mechanosensitive cells could be found along the entire length of the cochlea with functional mechanotransduction in nearly 100% of the cells from the base and mid basal quarter and $>80\%$ of the cells in the mid apical quarter and apex (Fig. 4A). Although the data revealed a slight decline in the percentage of transducing cells in some of our sample sets at later stages (Fig. 4A), we suspect that the decline does not reflect early-onset, age-related hearing loss but is more likely the result of hair cell damage induced during excision of the cochlea from the bulla at later stages when there is more ossification. Because the percentage of mechanosensitive cells must vary during development between zero and some maximal percentage, we opted to fit the rising phase with a Boltzmann equation. We found that the unconstrained Boltzmann fits had curves that paralleled each other for each of the four cochlear quadrants. Furthermore, the curves revealed a rapid rise in the acquisition of mechanotransduction such that the 10–90% rise occurred within $21 \pm 4.5$ (SD) h ($n=4$), similar to the rapid onset of transduction reported previously for mouse vestibular hair cells (Gélécoc and Holt 2003).

We also noted a systematic increase in the amplitude of the maximal transduction currents as a function of development between P0 and P2 in the base and P2 and P6 in the apex (Fig. 4B and Supplemental Table S2). In basal hair cells at $\geq$P3, there was a decrease in the maximal currents perhaps due to damage suffered during dissection of the older tissue; disruption of the unique fluid compositions of the cochlear spaces; and/or difficulties associated with stimulating the shorter basal hair bundles (Fig. 1). For this reason, we did not attempt to obtain data from cells in the basal portion after P5. In younger animals, the largest transduction current we recorded was from a basal P2 outer hair cell and its amplitude was $-689$ pA at a holding potential of $-64$ mV. Assuming a reversal potential of 0 mV, this corresponded to a conductance of 10.8 nS, which would predict a maximal current of $-904$ pA at a holding potential of $-84$ mV, similar to the maximal currents reported in rat basal outer hair cells at P4 (Waguespack et al. 2007). Our largest average currents were also obtained at P2 from outer hair cells at the base of the cochlea: $-468.7 \pm 42.6$ pA or $7.3 \pm 0.7$ nS ($n=9$).

Operating range and development

To quantify hair bundle operating range, bundles were deflected systematically with a range of step sizes up to $+1.25$ μm, and transduction currents were recorded. In a few cases at the most immature stages, we found that the current amplitudes did not saturate and thus we applied stimuli $\approx 2$ μm. Current-displacement [$I(X)$] relationships were generated from the peak transduction currents plotted as function of bundle displacement (Fig. 5) and were fitted using a second-order Boltzmann equation (Stauffer and Holt 2007). We noted a systematic change in the $I(X)$ curves as a function of development such that the curves were broader at early stages and became steeper over the subsequent few days. This pattern held true for cells from both the base and apex as illustrated for representative cells in Fig.
A previous report from P6 to P8 apical mouse outer hair cells of 0.44 μm (Stauffer and Holt 2007). Because the stimulus pipette can impose variable bias in the resting position of the hair bundle, we did not attempt to quantify the amplitude of the transduction current active at the bundle’s rest position.

**Adaptation and development**

At the developmental onset of mechanotransduction in outer hair cells, we noted that sustained hair bundle deflections evoked currents that decayed or adapted to a steady-state level within 50–60 ms. The current decay was best described by a double-exponential equation that suggested that both fast and slow components of adaptation were present at the onset of transduction. The total extent of adaptation was 75–85% on average at the onset of transduction, and we never observed transduction currents that lacked adaptation, suggesting that adaptation is integral to mechanotransduction in mouse outer hair cells. However, we did note some refinement in the properties of adaptation as a function of development. To quantify the properties of adaptation, we analyzed the raw current traces that had peak currents equal to ~50% of the maximal transduction current. Because the time course of the current decay at the mid-point of the \( R(X) \) relation mirrors the adaptive shift of the \( R(X) \) relation, analysis of the 50% current provides a good measure of both aspects of adaptation (Stauffer and Holt 2007). Representative traces are shown from P0 to P4 basal outer hair cells in Fig. 6A and from P2 to P6 apical cells in B. Each current trace was fitted with a double-exponential equation, which allowed an estimate of the fast and slow time constants of adaptation. Fast time constants are plotted as a function of development in Fig. 6C for 36 basal cells and 37 apical cells. The data were superimposed with sigmoidal curves to facilitate visualization of the trend. We noted a decrease in the time constants (i.e., faster adaptation) a few days after the onset of transduction. At the onset of mechanosensitivity, basal hair cells had fast time constants of 2.0 ± 0.4 ms (n = 10) at P0 that decreased to 0.45 ± 0.15 ms (n = 7) by P4. In apical hair cells, fast adaptation was slower with time constants of 3.7 ± 0.8 ms (n = 3) at P2 but that showed a parallel decrease to 1.1 ± 0.3 ms (n = 7) by P5 and 0.9 ± 0.1 ms (n = 3) by P8. The smallest fast time constant we measured was 81 μs, evoked by a small step displacement (200 nm) in a P4 basal outer hair cell, which was in the same range as fast adaptation in rat outer hair cells (Kennedy et al. 2003). We also noted a similar developmental trend for the slow time constants which were 21.6 ± 6.3 ms (n = 10) in the base and 38.6 ± 10.3 ms (n = 3) at the apex at the onset of transduction. As development proceeded, slow adaptation became faster in both the base and the apex. Slow adaptation stabilized after P4 in the base and P6 in the apex with time constants of 10.7 ± 3.1 ms (n = 7) and 8.5 ± 1.5 ms (n = 9), respectively. The extent of adaptation, taken as the amplitude of the current decay relative to the peak current, was also analyzed during development. While variations were observed within a given stage and position along the cochlea, our analysis showed that adaptation became more complete as cells developed (Fig. 6E). This trend was particularly evident in the apex where the extent of adaptation averaged 74% at P2 and reached 93% after P6. Although less prominent, a similar trend was observed in basal hair cells.
Expression of hair cell genes during development

The spatiotemporal gradient of hair cell mechanotransduction acquisition charted in the previous sections presents an opportunity to screen for hair cell genes with mRNA expression patterns that parallel or precede the pattern of functional development. In particular, we screened the spatiotemporal mRNA expression pattern of hair cell genes that may be involved in hair bundle development and/or mechanotransduction. Genes with mRNA expression onsets that occur after the onset of mechanotransduction can be excluded from the list of candidate transduction molecules, whereas genes the expression of which precedes or parallels the transduction onset may be worthy of further consideration.

Total RNA was extracted from tissue harvested from four quarters of the developing mouse cochlea that included both sensory and nonsensory cells at seven developmental stages between E17 and P8 (see METHODS for details). We used quantitative RT-PCR to examine the spatiotemporal mRNA expression pattern for seven putative hair bundle/mechanotransduction genes. Prior work has shown that the protein products for each of the seven genes have been immunolocalized to cochlear hair bundles. In particular, we examined four unconventional myosins, myosin 1c (Myo1c), myosin 3a (Myo3a), myosin 7a (Myo7a), and myosin 15a (Myo15a) as well as the putative tip-link molecules protocadherin 15 (Pcdh15) and cadherin 23 (Cdh23) and the calcium pump PMCA2 (Atp2b2). We also examined expression of another hair cell gene, β actin (Actb), as an internal control that was measured in each sample and was derived from the same amount of RNA (see METHODS). Actb expression levels appeared quite consistent among the 28 samples (Fig. 7A; Supplemental Table S3) validating its use as an internal control. As such, the expression of each gene of interest was normalized to the Actb expression level for each of the 28 samples to yield relative expression. To validate our protocols for RNA extraction and quantitative assessment of expression levels of hair cells genes, we began with an examination of the outer hair cell-specific gene, prestin (Slc26a5), the expression of which has been well characterized previously (Belyantseva et al. 2000; Gross et al. 2005). Figure 7B reveals that there was a sharp rise in Slc26a5 expression that began at P4 and that...
coincided with the rise in prestin expression and preceded the onset of somatic outer hair cell motility (Belyantseva et al. 2000). That the general spatiotemporal trend in Slc26a5 expression paralleled that reported previously, raised confidence that our protocol and analysis could yield meaningful mRNA expression profiles for functionally relevant hair cell genes. While the data revealed sample-to-sample variation (Fig. 7; Supplemental Table S3), in the following text we focus on the general spatiotemporal trends in gene expression that emerged.

We began with an examination of the mRNA expression patterns of Myo3a, Myo7a, and Myo15a because all three proteins have been localized to hair bundles (Belyantseva et al. 2003; Hasson et al. 1995; Liang et al. 1999; Schneider et al. 2006) and have been implicated in bundle development and because mutations in all three have been associated with hair cell dysfunction and deafness (Gibson et al. 1995; Self et al. 1998; Walsh et al. 2002; Wang et al. 1998; Weil et al. 1995, 1997). We also examined Myo1c because the protein product has been shown to participate in adaptation in mouse vestibular hair cells (Holt et al. 2002; Stauffer et al. 2005) and has been localized to hair bundles of mouse auditory hair cells (Dumont et al. 2002). Myo3a, Myo7a, and Myo15a (Figs. 7C-E) showed a tonotopic gradient of expression at E17 with the lowest mRNA expression levels in the apex and progressively higher expression levels toward the base. Myo3a expression level at P0 parallels in situ hybridization data from basal cochlear hair cells of the newborn mouse (Walsh et al. 2002).

A rapid rise in the expression of Myo3a, Myo7a, and Myo15a from E17 to P0–P2 was observed in the apical half such that by P2 mRNA expression levels became more uniform across the entire cochlea. For all three myosin mRNAs, the rise in expression preceded the spatiotemporal onset of mechanotransduction by several days. A gradual inversion in the tonotopic gradient of expression of these three myosin mRNAs was observed such that by P8 higher expression was detected in the apex than in the base. Myo1c, on the other hand, had a base to apex spatiotemporal mRNA expression pattern that was correlated with the onset of mechanotransduction (Fig. 7F). We generated a scatter plot with the mean transduction current from Fig. 4B plotted as a function of Myo1c expression taken from Fig. 7F. Data were fitted with a linear regression that demonstrated a highly significant correlation ($P < 0.0001$) between current amplitude and expression level of Myo1c with a correlation coefficient ($r$) of 0.85 (Supplemental Fig. S1A). Comparison of acquisition of transduction (Fig. 4A) and Myo1c expression (Fig. 7F) across all regions of the cochlea showed that, on average, Myo1c expression preceded the development of mechanotransduction by 18 ± 9.1 h. Similar analysis revealed no statistically significant correlation between the onset of transduction and mRNA expression of any of the other myosins examined, Myo3a ($P = 0.25$; $r = -0.25$), Myo7a ($P = 0.42$; $r = -0.18$), and Myo15a ($P = 0.073$; $r = -0.38$). Of course, a lack of correlation should not be taken to mean that these myosins are not involved in the development of hair cell transduction. Because their expression preceded the onset of transduction, they remain viable candidates for roles in transduction and development. Likewise, because Myo1c expression also preceded the onset of transduction, its potential role in transduction development is boosted by the correlation we report here. Nonetheless, the correlation does not imply a causal relationship, and therefore involvement of myosin 1c in
mechotransduction and adaptation in auditory hair cells will require further investigation.

We also investigated mRNA expression for the putative tip-link molecules cadherin 23 and protocadherin 15 (Kazmierczak et al. 2007). The Cdh23 primer set used for this experiment recognized all known isoforms, whereas Pcdh15 primer sets were specific for the candidate tip-link isoform, CD-3 (Ahmed et al. 2006; Haywood-Watson 2006). The pattern of Cdh23 expression was similar to the tonotopic pattern of Myo3a with low expression at the apex and high expression at the base at E17 (Fig. 7G). Although the gradient remained during the first postnatal week, it became less steep as mRNA expression levels rose in the apex and declined in the base. In contrast to the other molecules examined here, Pcdh15 expression showed a markedly different pattern with little tonotopic distribution. There was high Pcdh15 expression at embryonic day 17, which declined uniformly across the cochlea as a function of development (Fig. 7H). The initial high Pcdh15 expression followed by a decline suggests an important role early in development and that only low, perhaps maintenance level, mRNA expression is required for normal function at later developmental stages. That the spatiotemporal patterns of Cdh23 and Pcdh15 show little resemblance to each other suggests that these molecules may have other functions in addition to their putative role as components of the tip link. Furthermore, the lack of similarity between the Cdh23 and Pcdh15 expression pattern suggests that their expression is likely regulated by different mechanisms.

Last, we examined the mRNA expression of the calcium pump, PMCA2 with primers that were designed to recognize the hair bundle specific isoform, PMCA2a (Dumont et al. 2001) (Fig. 7I). Interestingly, we noted a spatiotemporal rise in PMCA2 mRNA expression that preceded but was loosely correlated with the refinement of several properties of transduction and adaptation (Supplemental Fig. S2), including the change in adaptation time constants and extent (Fig. 6, C–E) and the decrease in operating range (Fig. 5C). As such, we propose that a spatiotemporal gradient in the calcium buffering capacity of the hair bundle, mediated in part by PMCA2a, and probably other calcium buffers, may contribute to the maturation and refinement of the properties of mechanotransduction and adaptation in mouse outer hair cells.

DISCUSSION

Here we present the first characterization of a spatiotemporal gradient in the acquisition of transduction in outer hair cells of the mouse cochlea. The onset of transduction, from base to apex over the first postnatal week, was examined by analysis of FM1-43 uptake in acutely excised cochlea and by electrophysiological recordings of transduction currents in outer hair cells. FM1-43 uptake increased gradually from P0 at the base to P7 in the apex. Consistent with our quantitative measurements, Gale et al. (2001), who examined FM1-43 uptake in mouse cochlear hair cells between P2 and P4, noted a gradient in the uptake of FM1-43 with greater fluorescence in the base at P4. We found that the onset of FM1-43 uptake at the basal end at P0 coincided with the onset of mechanotransduction as assayed by measurement of transduction currents. Because we observed no FM1-43 uptake and no transduction current at developmental stages prior to P0, we conclude that the earliest acquisition of mechanosensitivity in the mouse cochlea was P0, several days later than the onset of mechanotransduction in the mouse utricle hair cells (Géleoc and Holt 2003). Given that the functional development of vestibular system precedes that of the auditory system, this result is not surprising.

Interestingly, the onset of mechanotransduction in the apical hair cells of the mouse cochlea occurred prior to the onset of FM1-43 uptake by a couple of days. At P2, we were able to evoke transduction currents from hair cells in all regions of the cochlea. That transduction was present at P2–P3 in hair cells from the apex while FM1-43 uptake did not rise in the apex until P4–P6 suggested that following the acquisition of transduction, there was a gradual leftward shift in the position of the IX relation that resulted in a rise in the resting open probability. We did not measure changes in open probability at rest electrophysiologically due to the variable bias in bundle position that can be induced during placement of the stimulus pipette. However, we did note that in apical samples which lacked FM1-43 uptake but had already acquired mechanosensitivity, hair bundles could be deflected to induce FM1-43 uptake (data not shown), consistent with the presence of functional mechanotransduction channels that were closed at rest. In chick auditory hair cells, Si et al. (2003) found a bidirectional change in FM1-43 uptake as a function of development that they also suggested reflected developmental changes in the open probability at rest.

We observed a rapid rise (~21 h) in the number of mechanosensitive outer hair cells for each cochlear location beginning at P0 in the base and extending to the apex by P2–P3. The rapid rise was consistent with that reported by Géleoc and Holt (2003) for vestibular hair cells (~18 h). Furthermore, in both mouse vestibular (Géleoc and Holt 2003) and mouse auditory hair cells we found that all the properties of mature transduction were present at the onset of transduction including directional sensitivity, a sigmoidal stimulus response relationship and both fast and slow adaptation. Unlike mouse vestibular cells, but similar to chick auditory hair cells (Si et al. 2003), we found that mouse outer hair cells exhibited a gradual refinement in several transduction properties over the few days that followed the onset of transduction. The refinements included an increase in the amplitude of the mean maximal transduction currents, a narrowing of the IX relation, a decrease in the time constants of fast and slow adaptation and an increase in the extent of adaptation. We did not observe the discrete “step-wise” acquisition in the properties of transduction recently reported for rat auditory hair cells (Waguespack et al. 2007). Based on the rapid acquisition of transduction with all the qualitative features of mature transduction and the subsequent refinement, we present the following model for development of transduction in the mouse cochlea. Because we never saw transduction without adaptation, we suggest that the molecular correlates of these functions probably interact closely and are both present at the developmental onset of transduction. Because the slow adaptation motor is hypothesized to move along the actin core of the bundle, it is attractive to speculate that it may serve a second function, namely, transport of the transduction apparatus to the tips of the stereocilia during development. We suggest that once in place at the tips of the stereocilia, a single transduction unit becomes functional with all the qualitative properties of mature transduction. The subsequent maturation and refinement in the quantitative properties of...
transduction may result from a number of continuing developmental mechanisms including insertion of additional transduction units, continual development of hair bundle morphology and developmental changes in the calcium buffering capacity of the hair bundle.

To explore the contributions of hair bundle morphology to transduction maturation, we analyzed SEM images of hair bundles from outer hair cells between P0 and P6 and noted several gradual changes over the first postnatal week, including growth in hair bundle height at the apical end and, curiously, a significant reduction in bundle height at the basal end. A reduction in bundle height has been described previously in rat outer hair cells (Roth and Bruns 1992). While a decrease in bundle height in basal hair cells may account for the decrease in operating range, it is unlikely the only factor since operating range stabilized in the base after P2 while bundles continued to shorten throughout the first postnatal week. Furthermore, we noted that bundles grew taller in the apex while at the same time the operating range in those cells decreased dramatically. As such, morphological changes in the bundle may contribute to refinement in some properties of transduction but cannot be the only factor.

Another possible explanation for the developmental refinement of transduction properties arises from the spatiotemporal changes we observed in the mRNA expression of the hair bundle calcium pump, PMCA2. We hypothesize that changes in resting hair bundle calcium concentration, in turn the result of developmental changes in calcium buffers (Hackney et al. 2005) and calcium pumps, may contribute to the refinement in the quantitative properties of transduction and adaptation over the first postnatal week. In particular, the time course and extent of fast and slow adaptation are likely to be directly affected. With higher expression of calcium buffers and pumps, resting bundle calcium concentrations would drop, which would tend to increase adaptation and in transduction development in outer hair cells of mouse outer hair cells. (Holt et al. 2002; Stauffer et al. 2003) showed the earliest detectable myosin 1c immunostaining coincided with the acquisition of transduction in rats and chicks, respectively.

The spatiotemporal patterns of expression that we observed for other myosins cannot exclude those molecules from further consideration for a role in transduction, development, and adaptation. In particular, we saw a dramatic rise in the tonotopic expression pattern of Myo3a beginning at E17, suggesting that it may play a role in hair bundle development prior to the onset of transduction. However, it should be noted that our cDNA samples included both sensory and nonsensory cells, so localization of message or protein in hair cells is required to invoke specific hair bundle functions. Myosin 3a protein has been immunolocalized to the tips of outer hair cell stereocilia in rats and mice, but like myosin 1c, the peak in immunofluorescence occurred between P6 and P10 (Schneider et al. 2006) well after the onset of transduction and adaptation in rat outer hair cells (Waguespack et al. 2007) and well after the onset in mouse outer hair cells.

That expression of Cdh23 and Pcdh15 both preceded the onset of transduction is consistent with a role for these molecules in the formation of tip links (Kazmierczak et al. 2007). Interestingly, we noted high expression of both Cdh23 and Pcdh15 at E17, the same stage at which Goodyear et al. (2005) reported the appearance of tip links. Although neither the appearance of tip links nor the expression of Cdh23 and Pcdh15 were correlated with onset of hair cell transduction we report here, it is possible that tip links may develop before the onset of functional transduction. Alternatively, of the multiple Cdh23 splice forms (Lagziel et al. 2005), analysis of the expression pattern of the tip-link splice form may reveal a pattern that more closely resembles the onset of transduction.

Based on our morphological, physiological, and molecular characterization, we suggest that the developmental acquisition of mechanotransduction in mouse cochlear outer hair cells follows a stereotyped pattern beginning ~1 wk after the onset of Atoh1 expression. For example, if Atoh1 expression begins at about embryonic day 13 in basal hair cells (Chen et al. 2002), the onset of mechanotransduction begins at the equivalent of E20 or P0. At this stage, all the properties of hair cell transduction and adaptation are present, at least qualitatively. The amplitude of the currents grow as more transduction units are transported to the site of transduction near the tips of the stereocilia, perhaps carried by myosin 1c or other hair bundle myosins. Over the subsequent few days, expression of PMCA2 rises, which we suggest leads to a decline in intracellular calcium levels in the hair bundle and a refinement in the quantitative properties of transduction including operating range and the time course and extent of fast and slow adaptation until they reach their mature values. We find that the same stereotyped pattern of development progresses in a gradient or wave of functional

ACQUISITION OF TRANSDUCTION IN OUTER HAIR CELLS

J Neurophysiol • VOL 101 • JUNE 2009 • www.jn.org
development from the base toward the apex. In a similar manner, acquisition of mechanotransduction in apical outer hair cells begins ~1 wk after the onset of Atoh1 expression (~E15) in those cells or at the equivalent of ~P2 where it is also followed by a rise in expression of PMCA2 and a subsequent refinement in the properties transduction and adaptation.

Last, we suggest that future experiments designed to test the function of candidate transduction molecules may benefit from the developmental timeline presented here and, furthermore, that the promoter sequences for these genes may be engineered to drive precisely defined spatiotemporal patterns of expression for other genes of interest.

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