Research Article

Spatial Gradients in the Size of Inner Hair Cell Ribbons Emerge Before the Onset of Hearing in Rats

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ABSTRACT

The size and locations of pre-synaptic ribbons and glutamate receptors within and around inner hair cells are correlated with auditory afferent response features such as the spontaneous discharge rate (SR), threshold, and dynamic range of sound intensity representation (the so-called SR-groups). To test if the development of these spatial gradients requires experience with sound intensity, we quantified the size and spatial distribution of synaptic ribbons from the inner hair cells of neonatal rats before and after the onset of hearing (from post-natal day (P) 3 to P33). To quantify ribbon size, we used high resolution fluorescence confocal microscopy and 3-D reconstructions of immunolabeled ribbons. The size, density, and spatial distribution of ribbons changed during development. At P3, ribbons were densely clustered near the basal/modiolar face of the hair cell where low SR-groups preferentially contact adult hair cells. By P12, the disparity in ribbon count was less striking and ribbons were equally likely to occupy both faces. At all ages before P12, ribbons were larger on the modiolar face than on the pillar face. These differences initially grew larger with age but collapsed around the onset of hearing. Between P12 and P33, the spatial gradients remained small and began to re-emerge around P33. Even by P12, we did not find spatial gradients in the size of the post-synaptic glutamate receptors as is found on afferent terminals contacting adult inner hair cells. These results suggest

that spatial gradients in ribbon size develop in the absence of sensory experience.

Keywords: auditory nerve, ribbon synapses, glutamate receptors, calretinin, innervation patterns, development

INTRODUCTION

The entire input to a mature mammalian type I auditory neuron is provided by a single ribbon synapse formed between an inner hair cell and an afferent neuron. But, any one inner hair cell has many ribbons of different sizes, each providing input to afferent neurons that individually differ in their response properties. Some neurons are most sensitive to low sound intensities and have high rates of spontaneous discharge (high-SR) whereas others are most sensitive to high sound intensities and have low rates of discharge (low-SR) (Liberman 1982). Since the combined activity of these so-called “SR-groups” is important for representing a wide dynamic range of sound intensities, understanding the timeline over which these features emerge is crucial for understanding the development of sound intensity encoding.

In adult inner hair cells, the size of a synaptic ribbon opposing an afferent terminal varies depending on the response properties of the target neuron. In cats, ribbons opposing low-SR/high-threshold neurons are larger than those opposing high-SR/low-threshold neurons (Merchan-Perez and Liberman 1996). Large, vesicle rich ribbons are predominantly found near the modiolar face (neural side) of the inner hair cell where low-SR fibers preferentially

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terminate and small ribbons are found on the pillar face (abneural side) where high-SR fibers terminate (Liberman et al. 2011). Consistent with the idea that ribbon size is associated with the response of its partner neuron, modiolar-to-pillar gradients in ribbon size are particularly prominent in the mid-frequency regions of the mouse cochlea where the greatest diversity in afferent spontaneous rates has been observed (Taberner 2005; Yin et al. 2014). Spatial gradients in ribbon size are seen in various species, including cats, mice, and guinea pigs (Merchan-Perez and Liberman 1996; Liberman et al. 2011), suggesting that ribbon size is generally a useful marker for functional diversity.

Although a qualitative association between ribbon size and SR-groups has been observed in several species, it is not clear how ribbon morphology contributes to differential encoding of sound intensity. Indeed, the association between ribbon size and afferent response in auditory afferents is counter to our expectation based on other systems; in the retina, synapses with large ribbons have the fastest rates of synaptic release (e.g., Mehta et al. 2013), whereas in the cochlea, large ribbons oppose the low-SR-group. Thus, the functional properties of ribbon-bearing synapses likely rely on combinations of several pre- and post-synaptic mechanisms. Several pre-synaptic features could contribute to differential encoding of sound intensity. These features include spatial gradients in the size, placement, and biophysical properties of the calcium currents that trigger exocytosis (e.g., Wong et al. 2014a; Ohn et al. 2016), as well as differences in the likelihood that such exocytosis produces mono-phasic versus multi-phasic EPSCs (Grant et al. 2010). In zebrafish, ribbon size and the localization of calcium channels also appear to be reciprocally regulated; the ribbon-specific protein RIBEYE appears to direct the localization of Cav1.3 calcium channels whereas Cav1.3 channels appear to regulate the size of synaptic ribbons (Sheets et al. 2011, 2012). Post-synaptic differences such as glutamate receptor type and density (Liberman et al. 2011) and ion channel heterogeneity (e.g., Liu and Davis 2007) may also contribute to the differential response properties of spiral ganglion neurons. Whether ribbon size is causally or consequentially related to differential synaptic function is a matter of debate (a recent study by Kantardzhieva et al. (2013) argues for the latter). Nevertheless, ribbon size is strongly correlated with afferent neuron sensitivity to sound intensity. Here, we sought to characterize the developmental timeline over which such spatial gradients in ribbon size emerge in rats.

Neonatal rats have been commonly used for studying the electrophysiology of hair cells, synaptic release, and afferent terminals (e.g., Glowatzki and

Fuchs 2002; Grant et al. 2010; Yi et al. 2010; Rutherford et al. 2012), but the temporal sequence over which their synapses take on spatially distinct features has not been characterized. In this study, we quantified the spatial distribution and size of synaptic ribbons and post-synaptic glutamate receptor clusters by fluorescence confocal microscopy in early post-natal rats. We also compared the timeline for synaptic development to changes in the overall pattern of innervation. By quantifying the spatial distribution and size of ribbons and glutamate receptors during the period when significant synaptogenesis and refinement occurs, we establish a time line for the functional differentiation of the auditory nerve.

We show that spatial gradients in synaptic ribbon size are already present in immature inner hair cells well before the onset of sensory and conductive hearing, and thus likely to develop independent of experience with sound intensity. However, the gradients in glutamate receptors, that are expected in the mature animals, do not develop by the onset of hearing, leaving open the possibility that post-synaptic differentiation is susceptible to sensory input.

METHODS

Preparation

Temporal bones in rats from post-natal (P) day 3 through P63 were dissected in chilled and oxygenated Leibovitz-15 (L-15) medium supplemented with 10 mM HEPES (pH 7.4, ~315 mmol/kg). Between 3 and 7 cochleae contributed to the data set at most ages. One sample was examined at P63, but the immunolabeling for CtBP2 was not bright enough to quantify.

Dissection began by removing the bony covering of the otic capsule to reveal three turns of the cochlea. Sharp scissors were used to separate the apical, middle, and basal turns. Our analyses focused on the middle cochlear turn where gradients in ribbons size and spontaneous rates are most striking (Yin et al. 2014). The stria vascularis and other overlaying membranous layers including the tectorial membrane were removed using fine forceps. Animals older than P12 were euthanized and intra-cardially perfused. The otic capsule was removed, the apical cap was cracked, and the whole cochlea was perfused through the basal turn for 15 min with chilled 4% paraformaldehyde. Next, cochleae were dissected into three turns (either before or after decalcification for 2 to 3 days in EDTA). Then each cochlear turn was processed for immunolabeling as described below.

Immunohistochemistry

Cochlear samples were attached to a glass coverslip using the tissue adhesive CellTak (Corning) and placed in a culture well. Immobilizing the sample prevented damage during the solution transfers needed for the fixation, wash, and incubations steps of the immunolabeling procedure. All subsequent immunolabeling steps were performed in the same well with the samples so immobilized.

Next, samples were fixed in 4 % paraformaldehyde for 15 min at room temperature followed by three rounds of gentle washing in phosphate-buffered saline (PBS). Then, the preparation was incubated for 1 h at room temperature in a blocking/dilution buffer consisting of 16 % normal goat serum, 0.3 % Triton X, 450 mM NaCl, and 20 mM phosphate buffer. All primary and secondary antibodies were diluted at a ratio of 1:200 in the above dilution buffer. Preparations were incubated in dilutions of primary antibodies at 4 °C for between 16 and 20 h. Incubations in Alexa Fluor-conjugated secondary antibodies were for 1 h at room temperature. Following the primary and secondary incubations, we washed the samples for three 15 min rounds in 1× PBS.

The following primary antibodies were used to label the following structures: (1) anti-CtBP2 mouse monoclonal IgG (Becton Dickinson Company) to label the synaptic ribbon-specific protein RIBEYE, (2) anti-myosin-VI-rabbit poly clonal (Proteus Bioscience Inc.) to label the cytoplasm of inner hair cells, (3) anti-GluR2/3 rabbit poly clonal IgG (Millipore) to label post-synaptic receptors, (4) anti-beta III tubulin mouse IgG2a (TUJ1; Covance) to label all cochlear neurons, (5) anti-calretinin rabbit polyclonal (Swant 7699 or Swant 7697) to label cochlear afferents and hair cells, and (6) phalloidin to label hair bundles. The following secondary antibodies acquired from Life Sciences Inc. were used: Alexa Fluor 488 anti-mouse, Alexa Fluor 594 anti-rabbit, and Alexa Fluor 633 anti-goat. Samples at different ages were typically processed on different days when animals of the correct age became available. Samples from several experiment days were pooled to provide the data at any particular age.

Imaging

Fixed and immunolabeled samples were mounted under a glass coverslip and onto glass slides with the fade-protectant medium Vecta-shield (Vector Products). Hardened nail polish dots were placed under the coverslip to prevent it from crushing the cochlear sections.

We generated a series of z-stack images of immunofluorescent signals using an Olympus FV1000 laser

scanning confocal microscope with a 60×, 1.42 numerical aperture, oil immersion objective, with a threefold zoom. The scanning format was set to collect 1024 × 1024 pixels yielding a sampling resolution of 0.069 μm/pixel in the lateral dimension (x-y). Sections were acquired with z-steps of 0.49 μm with pinholes set at 1 Airy unit. To minimize photo bleaching, we limited high-resolution scans to the very basal pole of the hair cells where the cell nucleus, ribbons, and glutamate signals were confined.

In samples with more than one fluorescent signal, each channel was scanned in sequence with the same optical section thickness. Laser power, gain and offset settings for each channel were carefully set to optimize the dynamic range of the fluorescent signal in each sample (between 0 and 4095). Because the brightness of the fluorescent signal can vary from sample to sample, here, as in other similar studies (e.g., Liberman et al. 2011; Yin et al. 2014), estimates of relative ribbons sizes within individual samples are likely more robust than estimates of absolute ribbons sizes. As a result, in most figures, volume estimates based on the fluorescent signal were compared across samples only after normalizing by the median value within each sample (e.g., Liberman et al. 2011).

Analysis

3-D reconstructions and volumetric analysis of CtBP2- and GluR2/3-labeled structures were done offline using the *Surface Object* toolbox of the image processing program Imaris (Bitplane by Oxford Instruments). In a preliminary analysis, image segmentation and 3-D reconstruction was performed using a different program (Amira; Visage Imaging). Results were consistent across both software platforms. All quantification was based on the raw data without applying deconvolution filters.

The segmentation procedure was an iterative process in which the investigator selected thresholds for fluorescence intensity and parameters that defined the minimum size of objects (approximately greater than 10 voxels). The average threshold value was 470 ± 170 SEM (from a possible range of values from 0 to 4095). Based on these parameters, the surface rendering algorithm constructed a 3-D iso-intensity surface to represent each object (see Fig. 2). The volume of the surface object was proportional to the number of voxels encompassed by the surface. In several samples, we compared the integrated fluorescence intensity to the surface volume of the segmented ribbons. The integrated fluorescence intensity was computed as the sum of voxel intensities (greater than the threshold value) that were within the reconstructed surface. Because estimates of ribbon volume were highly correlated with estimates of

integrated fluorescence intensity ($r^2 = 0.98$), in most of the analyses, we reported ribbon size in terms of normalized volume.

The Cartesian coordinates of the center of mass and volume of each reconstructed surface object was exported as an excel spreadsheet that could be analyzed offline. We quantified the number and spatial distribution of ribbons within individual hair cells using custom programs in MATLAB.

Segmenting Ribbon Clusters. Sometimes ribbons were located in a dense cluster which the automatic segmentation detected as one large surface (Fig. 2). To separate such merged clusters, we exploited the stereotypical “tear drop” shape of individual ribbons (Fig. 2a) to fine-tune the parameters for the segmentation procedure. The tear drop shape results from the non-uniform 3-D point-spread function of the optics. Even in a tight cluster of ribbons, the potential boundaries of individual ribbons could be inferred from the tapering of the teardrops. Visual inspection of these fused elements allowed us to carefully “split” merged ribbons into individual objects. The extra care in segmenting was most important at P3 where ribbons were densely clustered. Although the strategy allowed us to identify and separate many merged objects, it is still possible that some large ribbons were actually closely clustered ribbons that we could not reliably resolve with the optical resolution of the system. Once a reasonable parameter set was defined it was applied uniformly to all the hair cells evaluated in the sample.

Statistics. Statistical significance was determined by subjecting the data to a two-way unbalanced analysis of variance (ANOVA) for age and spatial position. This was followed by a post hoc univariate analysis of variance at each age between P3 and P12 with a Bonferroni correction applied for multiple comparisons. Statistical analysis was performed in MATLAB and cross-checked by the software package JMP (SAS Institute Inc.).

Controls. In adult inner hair cells, opposing gradients in the size of GluR2/3 and CtBP2 puncta served as an internal control against potential scanning artifacts. This internal control was not possible in these immature animals because the immunolabel for glutamate receptors GluR2/3 formed a tight net around the hair cell rather than punctate plaques that are closely juxtaposed across from each ribbon. Moreover, gradients in the size of GluR2/3 plaques may be a developmentally acquired feature (as our data suggest); therefore, we could not assume that the opposing gradients in the size of the glutamate receptor would serve as an internal control against scanning artifacts. Instead, we ran control experiments in which we compared gradients in ribbon size after varying the sample mounting orientation and

scan directions. The purpose of these controls was to test if scanning artifacts due to differences in light penetration resulted in the ribbon size gradients.

To test if differences in light penetration/exposure influenced ribbon gradients, we quantified volumes in samples that were mounted in two orientations: with the hair bundles of outer hair cells facing up towards the coverslip or with the hair bundles facing down towards the coverslip. Since inner hair cells are tilted, the modiolar face of the inner hair cell was closer to the glass coverslip in the bundles up orientation, whereas the pillar face was closer in the bundles facing down orientation. Although the relative distance between the excitation light and ribbons was different in the two conditions, ribbons were larger on the modiolar face than on the pillar face in both conditions.

Next, we tested if differences light bleaching from different exposure times to the excitation laser could create size gradients as an artifact. Because the ribbons that are imaged later during the scan are exposed to the laser light for longer durations than those that are scanned earlier, we were concerned that they would experience more photo bleaching. Differential photo bleaching could erroneously create a size gradient in ribbon size where none existed. To test this, we scanned samples from basal to apical pole of the hair cell and vice versa. Regardless of scanning directions, the ribbons on the modiolar face of the inner hair cell were, again, on average larger than those on the pillar face (Fig. 3f).

Finally, in a subset of samples, we tested if defining ribbon size as integrated fluorescence intensity (see above for how we defined this measure), rather than volume, would yield different results. A linear regression analysis suggested that the two measures were highly correlated ($r^2 \sim 0.98$). Our general conclusion that modiolar-facing ribbons were larger than pillar-facing ribbons before P12 was not dependent on whether we defined ribbons size based on reconstructed volumes or integrated fluorescence intensity (see violin plots in Fig. 2g). We further tested if the brightest elements in the sample were biasing the statistics by comparing the modiolar to pillar ribbon sizes after eliminating the brightest elements (Fig. 2g; bracketed ribbons removed); the average ribbon size (volume or integrated intensity) was still larger on the modiolar face.

RESULTS

We describe the developmental timeline of inner hair cell synaptic structures and innervation in two parts. First, we describe developmental changes in synaptic

ribbon number, spatial distribution, and spatial gradients of ribbon volumes. Second, we describe the concurrent development of post-synaptic glutamate receptor plaques and cochlear innervation.

Developmental Changes in Pre-synaptic Ribbons

Ribbons were analyzed in whole mount preparations from the middle turn of cochleae in 35 rats ranging in age between P3 and P38. To characterize the development of pre-synaptic ribbons, we immunolabeled ribbons by using an antibody against the C-terminal binding-protein component of the ribbon-specific protein, RIBEYE (Schmitz et al. 2000; Maxeiner et al. 2016). As previously described, antibodies against CtBP2 brightly label synaptic ribbons and dimly label the nuclei of inner hair cells (Khimich et al. 2005). To visualize the distribution of ribbons relative to the orientation of the hair cells, we labeled the cytoplasm of the hair cell with myosin VI. Figure 1 shows example maximum intensity projections (in the x-y and x-z dimensions) of high-resolution confocal z-stacks from cochleae at different ages.

The Number and Distribution of Ribbons Within Inner Hair Cells. Bright spots corresponding to ribbons were found in both inner and outer hair cells at all ages (Fig. 1e, f). During the first post-natal week, the number and size of the CtBP2 plaques corresponding to ribbons in the outer hair cell were numerous with bright immunofluorescence. Although ribbons were visible in OHCs throughout the age range examined, their prevalence and size decreased dramatically as the animal aged (faint ribbons at P33 are marked by arrowheads in Fig. 1e, f).

The number and distribution of inner hair cell ribbons also changed during development (Fig. 1). At P3, ribbons were tightly clustered under and around the flanks of inner hair cells' nuclei. By P12, ribbons appeared more uniformly distributed around the circumference of the nucleus (Fig. 1d). Although there was little myosin VI immunofluorescence under the nucleus, by P33, the ribbon were located in a myosin VI-labeled area in the basal pole under the nucleus.

To quantify such age-dependent changes in numbers and spatial positioning, we analyzed ribbon size as an estimate of volume from 3-D reconstructions of fluorescently labeled CtBP2-enriched puncta. In some cases, the ribbons were closely positioned and the segmentation procedure required hand tuning to separate closely clustered ribbons into putative single ribbons (Fig. 2; see the “[Segmenting Ribbon Clusters](#)” section for more details). In some samples, we examined both the reconstructed ribbon volumes and the integrated fluorescence intensity (Fig. 2g; see the “[Controls](#)” section for more details); results

were similar for both methods of analysis. In any one sample, we analyzed all the ribbons from three adjacent inner hair cells per cochlear middle turn. Ribbon number per hair cell and position was defined in at least three animals (often 5–6 cochleae) per age. To reduce the variance in our assignment of modiolar versus pillar position, in each sample, we chose hair cells whose nuclei were well aligned in the x-y and x-z dimensions.

To demarcate the modiolar versus pillar sides of an inner hair cell, we drew a line through the center of the hair cells from its cuticular plate to basal pole (Fig. 1a–f). The orientation of the hair cell was defined either by antibodies against myosin VI (labels the cytoplasm of an inner hair cell) or phalloidin (labels the stereocilliary hair bundles). This axis line was extended in the z dimension to define a plane that bisected the hair cell into its modiolar and pillar halves. Since hair cell orientation changes as the tunnel of Corti opens during development (Roth and Bruns 1992), this bisecting plane was separately defined in each sample. In a few samples without co-labeled landmarks (e.g., Fig. 1e, f), the central axis was defined relative to the position of the inner hair cell's nucleus (faintly reactive to the CtBP2 antibody) and the CtBP2 reactive ribbons in the three rows of outer hair cells. Zoomed in slices of the basal pole of inner hair cells in the x-z dimension are shown in Fig. 2h–k. Transmitted light images (not shown) with a differential interference contrast (DIC) filter were additionally useful for verifying hair cell orientation.

We found a significant age-dependent decrease in the number of ribbons per hair cell. In Figure 3a, interquartile box plots show the total number of ribbons/hair cell and the number of ribbons on either the pillar or modiolar face. The overall number of ribbons per hair cell decreased with age (Fig. 3b); the average number of ribbons at P3 was nearly double to that at P12 (approximately 45 per hair cell down to 25 per hair cell (green symbols, Fig. 3b). Along with the decrease in overall ribbon numbers, the proportion of smaller ribbons was greater at P3 than at P12 (see histograms in Fig. 3e). Such age-dependent changes in ribbon numbers and volumes is consistent with previous observations in developing retina and cochlear hair cells, where small spherical ribbons in immature cells fuse to coalesce into the larger ribbons of the mature cell (Sobkowicz et al. 1982; Hermes et al. 1992; Wong et al. 2014b).

Spatial Gradients of Ribbon Size. Next, we compared the relative volumes of modiolar-facing and pillar-facing ribbons. Until about P12, larger ribbons were preferentially located about the modiolar face of the hair cell (Fig. 3b). Moreover, the relative difference in ribbon volumes increased systematically between P3

FIG. 1. Immunolabeling to visualize ribbons and hair cells. **a–d** Images are maximum intensity projections from confocal scans of cochlear middle turns of rats at P3, P6, P10, and P12. Samples are immunolabeled against CtBP2 to visualize ribbons and hair cell nuclei (green) and myosin VI to visualize hair cells (red). Note that the samples in **c** and **d** were mounted in the opposite orientation relative to **a** and **b** but all images in the x-z plane are oriented to show the apical pole of the hair cell at the top of the image. The

immunolabeled inner hair cell was used to define a plane to bisect the inner hair cell into pillar-facing and modiolar-facing halves. **e–f** Images at P16 and P33 were made without the antibody for myosin VI to label the cytoplasm of the hair cell. Yellow arrowheads show the position of ribbons in the outer hair cell. Scale bar applies to all images. Red bars adjacent to images indicate the scan area over which the maximum intensity projections were made.

and P10 (Fig. 3c). Unexpectedly, this trend collapsed by P12, when the volume differences between the modiolar and pillar faces was not as striking as it was for the preceding three time points (although the

difference was still statistically significant). The size difference between modiolar- and pillar-facing sides almost appears to reverse by P16, but, the reversal did not reach statistical significance ($p > 0.01$). By P33, a

FIG. 2. Analysis of ribbons. **a–c** Distantly spaced ribbons were easy to identify in the x-z plane as individual ribbons. Example segmentation based on the fluorescent scan (**a**) was used to determine the boundaries of the ribbon based on an intensity threshold (**b**). The ribbon surface was reconstructed to estimate volume (**c**). Note the non-uniform spread of light creates the tear drop shape characteristic of ribbons. **d–f**. Images through a cluster of ribbons from a P3 hair cell. Automatic segmentation often merged these clusters into one large surface. Careful segmentation based on the expectation that individual ribbons should appear as tear drops allowed us to split such merged clusters into smaller surfaces that we

expect to be individual ribbons. **g** Ribbon sizes (by integrated fluorescence intensity) for 173 ribbons from three P3 hair cells. Modiolar versus pillar ribbons are shown with accompanying accordion plots. Ribbon volumes are indicated by the gradient color. Modiolar-facing ribbons were larger than pillar-facing ribbons, even after eliminating the brightest/largest ribbons (*bracketed*); $p < 0.01$. **h–k** Zoomed in view shows basal pole of a hair cell with CtBP2-labeled ribbons (*green*) and nuclei (*faint green*) and myosin VI-labeled hair cells (*red*) at multiple time points. Nucleus is out of view at P38. Scale bar in **k** applies to the four panels from **h–k**.

small gradient in ribbons size with larger ribbons on the modiolar face appears to be emerging.

Previous studies in adult mice have shown that gradients in ribbon size are less striking in cochleae that are damaged by noise exposure. We do not think that damage was the reason that ribbon size gradients collapsed at P12 because we only analyzed samples in which all rows of inner and outer hair cells were visible with no obvious structural damage. In the worst-case scenario, our data included samples where the extreme edges of the cochlear turn showed some missing outer hair cells. Even in these samples, we only quantified ribbons in inner hair cells whose partner outer hair cells were clearly free of damage. Even so, when we further filtered our samples to only

include data from the most pristine preparations, the volume differences were not considerably different. In summary, these data show that there is a difference in ribbon size between the modiolar and pillar face as early as P3. This difference grows between P3 and P10 and experiences an unexplained reduction around P12. This collapse in ribbon size persists into the third post-natal week and appears to be re-emerging by around P33.

The spatial gradients in ribbon size we found were not a result of scanning artifacts. To control against light penetration/exposure artifacts, we quantified volumes in cochlear samples that were mounted and scanned in two orientations (Fig. 3f; see methods for details). Neither scanning direction nor mounting

◀ **FIG. 3.** Spatial distribution and quantification of ribbon volumes. The number, spatial distribution, and volume of ribbons changed during the first week of development. **a** Interquartile box plots showing the median, minimum, maximum, and interquartile ranges for the number of ribbons per hair cell (gray). Blue and orange box plots show the number of ribbons on the modiolar and pillar faces, respectively. Small x's and connecting lines represent mean values from each cochlea (5 cochleae contributed to the data at P3). **b** The total number of ribbons (green) per hair cell decreased with age and ribbons were less numerous on the pillar face (2-way ANOVA, $p = \{<0.001, 0.009\}$). Symbols show the mean $\pm$ SEM from 3 or more cochleae per age (number of cochleae are shown in the parenthesis below the symbol). Curves show a smoothed trend line through the data. At P3 and P6, more ribbons occupied the modiolar face (blue symbols) of the inner hair cell than the pillar face (red). By P9, the difference in spatial distribution was no longer significant and similar numbers of ribbons were found on both sides of the hair cell. p values for the modiolar versus pillar comparison were computed by a post hoc univariate analysis of variance with a Bonferroni correction to account for multiple corrections. Two asterisks indicate p values below 0.005; one asterisk indicates a p value less than 0.05. **c** Interquartile box plot for ribbon volume (normalized by the median value in each sample) from six middle turn cochleae at P6. Lines connect the data points belonging to the same cochlea. **d** Normalized ribbon volumes were, on average, larger on the modiolar face than on the pillar face, but this difference was age dependent (2-way ANOVA by age and position, $p = \{0.0015; <0.001\}$). Blue squares show the mean $\pm$ SEM ribbon volume for modiolar-facing (blue squares) and pillar-facing ribbons (red triangles). Between P3 and P12, modiolar-facing ribbons were larger than pillar-facing ribbons. **e** Histograms of ribbon volumes aggregated from all samples at P3 (red) and P12 (blue). Compared to the distribution of ribbon volumes at P12, a larger proportion of ribbons were very small at P3. **f** Controls confirm that spatial gradients in ribbon size are not scanning artifacts. *F.1* and *F.2* Schematic of samples mounted with hair bundles pointing towards the coverslip (triangle, $\wedge$) and away from the coverslip (squares). Scan direction from basal towards apical poles of hair cell (open symbols). Scan direction from apical towards basal pole of hair cell (closed symbols). *F.3*. Ribbon volumes estimated in three samples ($\wedge$, triangle, and square). The latter two with scan direction varied within the same sample (as described in *F.1* and *F.2*). Relative difference in ribbon volume was not dependent on scanning format.

direction influenced the general conclusion that modiolar-facing ribbons were on average larger than pillar-facing ribbons (Fig. 3f (*F.3*)).

Developmental Changes in Post-synaptic Receptors and Innervation

Previous studies on adult mouse cochleae reported that there are gradients in the size of post-synaptic GluR2/3 receptor plaques that oppose the gradients in pre-synaptic ribbons, with larger receptors plaques found on the pillar side of the hair cells and smaller plaques on the modiolar side (Liberman et al. 2011). It has been suggested that these gradients may contribute to the varied responses of the SR-groups and to their differential vulnerability to toxic noise exposure (Liberman et al. 2011; Furman et al. 2013). Since we found that size gradients for ribbons

emerged early during development, we wanted to see if there were concurrent changes in the spatial distribution and size of post-synaptic receptors and afferent fibers.

The Pattern of Glutamate Receptor Expression During Development. At P3 and P6, the labeling for GluR2/3 receptors formed a dense net-like pattern around the base of the hair cell (Fig. 5a, b, see also e for a zoomed in view). This dense labeling is consistent with previous reports from developing mouse cochleae (Huang et al. 2007; Sendin et al. 2007). Since the dendrites of individual type I SGN have many branches during early development (e.g., Echterler 1992), the thick net of labeling may correspond to each fiber making multiple glutamate receptor bearing contacts with individual hair cells. Here, the labeling was too dense in P3 and P6 animals to resolve individual pairings between ribbons and glutamate receptor patches (Fig. 5a, b). However, by P9 and P12, the labeling for GluR2/3 patches was more punctate so that juxtaposed pairings between CtBP2 and GluR2/3 patches were better resolved (more akin to the one-to-one pattern in adult cochleae) (Fig. 5c, d, see also zoomed view in e). Using 3-D reconstructions from the z-stack images of GluR2/3 patches, we quantified their volumes at P9 and P12 (Fig. 5f). Unlike the differences seen in pre-synaptic ribbon volumes, we did not find that the volumes of GluR2/3 patches differed between the pillar and modiolar faces.

Changes in Patterns of Afferent and Efferent Innervation Revealed by Calretinin and β -3 Tubulin Immunoreactivity.

Next, we examined how patterns of cochlear innervation changed between P5 and P13. We visualized nerve fibers with two antibodies: an antibody against β -3 tubulin to label all cochlear nerve fibers (Hallworth and Ludueña 2000) and an antibody against calretinin with the goal of labeling hair cells and type I afferent fibers (e.g., Liu and Davis 2014). At both P5 and P13, β -3 tubulin labeled the following structures: radial fibers crossing into the organ of Corti (rf), a dense network of fibers under the inner hair cell (ihc), tunnel crossing fibers (tc), and a dense network of fibers running under all three rows of outer hair cells (ohc1-3) (Fig. 5a, d). At P5, there were many fibers clustered about the modiolar face of the inner hair cell, and few fibers about the pillar face (Fig. 5g, compare the labeling density at p and m). By P13, we did not see such a striking disparity in labeling density between the two sides (Fig. 5f). This developmental change in the overall distribution of fibers is consistent with Figures 3 and 4 where we described that ribbons and glutamate receptors were also densely clustered about the modiolar face at early post-natal ages and become more uniformly distributed by P12.

FIG. 4. Development of post-synaptic glutamate receptors. **a–d.** Confocal images of cochlear samples from P3, P6, P9, and P12 rats. Samples are immunolabeled against GluR2/3 to visualize post-synaptic glutamate receptors (red) and against CtBP2 to visualize pre-synaptic ribbons (green). In one sample, phalloidin was used to label hair bundles. At P3, the labeling for GluR2/3 forms a dense net

around the basal pole of the hair cell. By P9 and P12, punctate pairings between individual CtBP2 and GluR2/3 patches were resolvable. **e** At P9 (open circles) and P12 (closed squares), GluR2/3 volumes were not different between the modiolar and pillar faces.

The labeling pattern for calretinin was only partially overlapping with the pattern for β -3 tubulin. Under the inner hair cells, calretinin labeled most but not all of the β -3 tubulin-enriched fibers (see merged images Fig. 5c and a zoomed version Fig. 5g). A group of

fibers that were enriched for β -3 tubulin, but not for calretinin ended in the neuropil around the modiolar face of the inner hair cell (see purple asterisk in Fig. 5c, g). Some of these fibers also followed a different pathway into the inner hair cell area than

FIG. 5. Developmental changes in cochlear innervation. Confocal images labeled to visualize innervation patterns at P5 (**a–c**) and P13 (**d–f**). **a, d** β -3 tubulin (green) widely labels radial fibers and fibers under the inner and outer hair cells. **b, e** Calretinin (red) labels hair cells and nerve fibers near the inner hair cell. **b** Under the outer hair cells only fibers under the first and second rows are labeled for calretinin at P5; **e** by P13 fibers that look like MOCs are labeled under all three rows (white arrowheads). **c, f** The merged image shows that the β -3 tubulin and calretinin signals are not completely

overlapping. **c** At P5, thin fibers that do not label for calretinin terminate in the neuropil about the modiolar face of the inner hair cell (double arrowheads and purple asterisk; also see **g**). **g** A zoomed in view of the merged image in **c**. **h** A cross-section through an inner hair cell at P13 showing a patch of fibers on the modiolar face that are not labeled for calretinin. Images in all panels are maximum intensity projections. Scale bar in **a** applies to **a–f**.

did the calretinin-enriched fibers (see double white arrow heads; Fig. 5c, g). By P13, we did not see such blind endings in the neuropil but still saw a patch of β -3 tubulin enriched but calretinin-poor fibers on the modiolar face of the inner hair cell (Fig. 5g; see purple asterisk in the cross-section). Although some of these calretinin-negative fibers could be olivocochlear efferents (which are known to be immunoreactive for β -3 tubulin; Hallworth and Ludueña 2000), we cannot rule out the possibility that some of them could be branches or filapodia of developing type I afferents. Consistent with this suggestion, calretinin is differentially enriched in the cell bodies of type I afferents with some cell bodies labeling more robustly for calretinin than others (Liu and Davis 2014).

The labeling patterns for calretinin and β -3 were also different under the outer hair cells. Calretinin-enriched fibers only reached the first two rows of outer hair cells at P5, but reached all the way to the third row by P13. In contrast, the β -3 tubulin-enriched fibers formed a thick network under all three rows of hair cells by P5, and this persisted to P13 (white arrows, Fig. 5c).

Our results suggest that calretinin is not an exclusive marker for type I afferents and hair cells but also labels the MOC efferents. Based on previous studies in mice, the type I projections into the outer hair cell regions should prune back to exclusively innervate the inner hair cells by the onset of hearing ($\sim$ P12) (Echteler 1992; Huang et al. 2007; Defourny et al. 2011). Here, the calretinin-selective fibers under the outer hair cells did not prune back by P13 but expanded out into the third row of outer hair cells (Fig. 5b, e, white arrowheads). This timeline suggests that the calretinin-enriched fibers under the OHCs at P13 are medial olivocochlear efferents (MOCs). In altricial rodents, MOCs innervate the cochlea in a progressive wave starting with axo-somatic contacts at the inner hair cell, moving into the first row of outer hair cells around P5 and reaching all three rows by the onset of hearing around P12 (Simmons et al. 1990, 1996; Simmons 2002; Bergeron et al. 2005). Also, consistent with their being MOCs, once the calretinin-enriched fibers reached the outer hair cell area, they turned in both apical and basal directions (Fig. 5e, yellow arrowheads). Taken together, these data suggest that the calretinin-enriched fibers under the outer hair cells at P13 (possibly also at P5) are likely to be those of the MOCs.

DISCUSSION

Cochlear synapses undergo many changes before the onset of hearing. Here, we described the develop-

mental timeline over which the distribution and spatial gradients in pre-synaptic ribbons, post-synaptic glutamate receptor plaques, and the patterns of cochlear innervation change in rat cochleae. Our results show a general timeline for synaptic maturation in inner hair cells where the number and distribution of synaptic ribbons and glutamate receptor plaques appears to be mature by the onset of hearing ($\sim$ P12). We found that modiolar-to-pillar gradients in ribbon size emerged as early as P3 but continued to change until well after the onset of hearing.

Timeline for Pre-synaptic Development

The number of synaptic ribbons per hair cell decreased during the first post-natal week and reached mature levels by P12. This developmental timeline is similar to that in mice where synaptic features such as decrements in ribbon numbers, size of calcium currents, coupling between calcium currents and exocytosis, and the biophysics of calcium dependent exocytosis are nearly mature by P12 (Beutner and Moser 2001; e.g., Sendin et al. 2007; Defourny et al. 2011; Huang et al. 2012; Johnson et al. 2013a). Moreover, we found that the proportion of very small ribbons is greater at P3 than at P12. This is consistent with the observation in mice that, between P3 and P12, ribbons coalesce from small spherical structures to larger plate-like structures (Sobkowicz et al. 1982; Wong et al. 2014a). Whether the changes in ribbon size, as assessed here by fluorescence confocal microscopy, truly reflect differences in absolute ribbon volume requires higher resolution imaging techniques (for discussion, see Rutherford 2015).

Modiolar-to-pillar gradients in ribbon size emerged by P3 (the earliest time point examined here), the difference peaked around P6 and then experienced a sudden decrease around the onset of hearing. After the onset of hearing modiolar-to-pillar ribbon gradients showed the first signs of re-emerging well after the third post-natal week. This surprisingly fluctuating timeline for spatial gradients is strikingly similar to that recently reported in mice (Liberman and Liberman 2016). We even saw a trend in which gradients in ribbon size appeared to reverse around P16, with larger ribbons on the pillar face and smaller ribbons on the modiolar face. Although we concluded that the trend did not reach statistical significance here, it is notable that a similar trend for reversal is also seen in mice. Overall, these results suggest that the temporal dynamics of synaptic development described here is not species specific and is robust to laboratory-specific variations in experimental technique and definitions of spatial position. Notably, a recent study in P14 mice also showed modiolar-to-

pillar gradients in ribbon size and calcium channel properties (Ohn et al. 2016). Of particular interest, they found that calcium currents were larger and had more depolarized thresholds on the modiolar face of the inner hair cell. The latter feature suggests a plausible mechanism by which inner hair cell synapses could be heterogeneously sensitive to sound intensity. Although our experiments do not extend out to full maturity, we expect that ribbon size gradients will fully re-emerge in mature rats, as they do in mice, because the auditory neurons of both species are similarly grouped by their spontaneous rates and threshold (Barbary 1991; Taberner 2005).

Mechanisms Driving Spatial Gradients

The early emergence of gradients in ribbons size suggests that this specialization is not driven by experience with sound. At P3, when we first saw gradients in ribbon size emerge, activity in the auditory nerve is largely driven by bursts of spiking from inner hair cells rather than by sound-driven graded receptor potentials (e.g., Kros 1998; Beutner and Moser 2001; Tritsch and Bergles 2010; Johnson et al. 2011). While the outer hair cells in mice are capable of transducing mechanical input as early as the first post-natal day, transduction currents are still immature (Waguespack et al. 2007). The first indication of sound-driven function in rats can be measured as bone conduction auditory brainstem responses (ABRs) sometime between P7 and P9 (Geal-Dor et al. 1993). Robust air conduction ABRs are only measurable around P12 (e.g., Jewett and Romano 1972; Geal-Dor et al. 1993). In cat, distinct SR-groups in the responses of auditory neurons are seen well after the onset of hearing (e.g., Walsh and McGee 1987). Taken together with this timeline, our results suggest that gradients in ribbon size (which eventually contribute to the encoding of a wide range of sound intensities) can develop in the absence of rich sensory experience. These results support the growing suggestion that such gradients are driven by polarized micro-environments around the inner hair cell (e.g., Graham and Vetter 2011; Yin et al. 2014).

A current hypothesis is that such micro-environments are created by proximity to olivocochlear efferents (Yin et al. 2014). Several recent observations support this suggestion. First, efferents are necessary for the normal development of inner hair cell synapses (Johnson et al. 2009, Johnson et al. 2013b). Second, there are complementary gradients in efferent terminal density such that the balance between large and small ribbons in any one hair cell is correlated with the relative distance between the efferents and the basal pole of the inner hair cell (Yin et al. 2014). Third, surgical or develop-

mental disruption of efferents collapses the modiolar-to-pillar gradients in ribbon and glutamate receptor sizes (Yin et al. 2014; Hickman et al. 2015). Given the fluctuating timeline for ribbon size shown here, the prediction is that efferent-driven gradients must be present very early in development and continue to change through the onset of hearing and beyond. There is considerable evidence supporting this suggestion. Medial olivocochlear efferents make temporary axo-somatic contacts with inner hair cells during early development but by the onset of hearing they almost exclusively innervate outer hair cells (Simmons et al. 1996; Bergeron et al. 2005; Huang et al. 2012). Similarly, the lateral olivocochlear efferent arrive at the inner hair cell a few days after the medial olivocochlear efferents and the balance between the two efferent systems continues to change through the onset of hearing (reviewed in Simmons 2002). The dynamic nature of efferent innervation during development may explain the serpentine timeline for ribbon gradients that we have characterized.

Timeline for Emergence of SR-Groups

Although gradients in ribbon size emerged as early as P3, we did not find opposing gradients in glutamate receptor size even by P12. These results suggest that the differentiation of the post-synaptic receptor plaques lags behind that of the pre-synaptic ribbons and may yet depend on sensory experience. Alternatively, size gradients in post-synaptic receptor plaques may have been too subtle during early development to resolve with the present techniques (even in adult mouse cochleae the size gradients in glutamate receptor patches are less striking than they are for synaptic ribbons (Liberman and Liberman 2016). Consistent with the suggestion that post-synaptic maturation may continue well after the onset of hearing, immunolabeling shows that the expression and distribution of ion channels at the spike-generator of rat auditory nerve fibers continues to mature till P30 (Kim and Rutherford 2016). Such post-synaptic immaturities could also account for the observation that the one-to-one fidelity between EPSPs and spikes that characterized adult guinea pig inner hair cell synapses (Siegel 1992) does not occur in rats for almost a week after the onset of hearing (Rutherford et al. 2012).

CONCLUSION

Ribbon synapses are found in many neuronal systems where rapid and sustained release of neurotransmitter is needed (e.g., retina, vestibular system, and lateral

line). Although the role of the ribbon itself in the mature function of the synapse is yet to be fully understood, emerging evidence suggests that it has a crucial role in synaptic formation and stabilization. In zebrafish lateral line, RIBEYE, a dominant component of the dense core of the ribbon, plays an important role in ribbon synapse formation by controlling the clustering of calcium channels and the formation of synaptic contacts (Sheets et al. 2011, 2012). Moreover, ribbon size and calcium channels appear to be reciprocally regulated (Sheets et al. 2012). These results are also consistent with the recent observation that number of calcium channels and their activation thresholds are correlated with ribbon size in mouse synapses (Ohn et al. 2016). The formation of synaptic contacts also appears to be independent of neurotransmission because synaptic contacts form even when vesicles are not filled with glutamate (Nagiel et al. 2009). Taken together, these studies suggest that the structure of the ribbon itself plays a crucial role in synaptogenesis.

The present finding that spatial gradients in ribbon morphology emerge within developing inner hair cells well before the onset of hearing suggests that this key anatomical marker for functional diversity is tuned early in development when mechano-electrical transduction (although present, Waguespack et al. 2007) is not fully mature. These results support the notion that micro-environments surrounding the inner hair cells lay the ground work for the eventual functional differentiation of auditory afferents. However, because gradients in glutamate receptor size—which have also been associated with functional diversity—did not develop in close synchrony with gradients in ribbon size, these micro-environments alone may not be sufficient for driving post-synaptic differentiation. Whether post-synaptic differentiation is explicitly tuned by sound-evoked activity remains to be determined. The importance of LOC efferents to maintaining spatial gradients in ribbon size and glutamate receptor plaques supports the suggestion that stimulus dependent feedback is important for maintaining functional diversity (Yin et al. 2014). Defining the timeline over which post-synaptic markers of afferent function (e.g., post-synaptic receptor size and subunit composition, afferent fiber diameter, and mitochondrial content) are acquired would be helpful in answering this question.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest The authors declare that they have no conflict of interest.

REFERENCES

- BARBARY AE (1991) Auditory nerve of the normal and jaundiced rat. I. Spontaneous discharge rate and cochlear nerve histology. *Hear Res* 54:75–90
- BERGERON AL, SCHRADER A, YANG D, OSMAN AA, SIMMONS DD (2005) The final stage of cholinergic differentiation occurs below inner hair cells during development of the rodent cochlea. *JARO - J Assoc Res Otolaryngol* 6:401–415
- BEUTNER D, MOSER T (2001) The presynaptic function of mouse cochlear inner hair cells during development of hearing. *J Neurosci* 21:4593–4599
- DEFOURNY J, LALLEMEND F, MALGRANGE B (2011) Structure and development of cochlear afferent innervation in mammals. *Am J Physiol Cell Physiol* 301:C750–C761
- ECHTELER SM (1992) Developmental segregation in the afferent projections to mammalian auditory hair cells. *Proc Natl Acad Sci U S A* 89:6324–6327
- FURMAN AC, KUJAWA SG, LIBERMAN MC (2013) Noise-induced cochlear neuropathy is selective for fibers with low spontaneous rates. *J Neurophysiol* 110:577–586
- GEAL-DOR M, FREEMAN S, LI G, SOHMER H (1993) Development of hearing in neonatal rats: air and bone conducted ABR thresholds. *Hear Res* 69:236–242
- GLOWATZKI E, FUCHS P (2002) Transmitter release at the hair cell ribbon synapse. *Nat Neurosci* 5:147–154
- GRAHAM CE, VETTER DE (2011) The mouse cochlea expresses a local hypothalamic-pituitary-adrenal equivalent signaling system and requires corticotropin-releasing factor receptor 1 to establish normal hair cell innervation and cochlear sensitivity. *J Neurosci* 31:1267–1278
- GRANT L, YI E, GLOWATZKI E (2010) Two modes of release shape the postsynaptic response at the inner hair cell ribbon synapse. *J Neurosci* 30:4210–4220
- HALLWORTH R, LUDUEÑA RF (2000) Differential expression of β tubulin isoforms in the adult gerbil cochlea. *Hear Res* 148:161–172
- HERMES B, REUSS S, VOLLRATH L (1992) Synaptic ribbons, spheres and intermediate structures in the developing rat retina. *Int J Dev Neurosci* 10:215–223
- HICKMAN TT, LIBERMAN MC, JACOB MH (2015) Adenomatous polyposis coli protein deletion in efferent olivocochlear neurons perturbs afferent synaptic maturation and reduces the dynamic range of hearing. *J Neurosci* 35:9236–9245
- HUANG L-C, THORNE PR, HOUSLEY GD, MONTGOMERY JM (2007) Spatiotemporal definition of neurite outgrowth, refinement and retraction in the developing mouse cochlea. *Development* 134:2925–2933

- HUANG L-C, BARCLAY M, LEE K, PETER S, HOUSLEY GD, THORNE PR, MONTGOMERY JM (2012) Synaptic profiles during neurite extension, refinement and retraction in the developing cochlea. *Neural Dev* 7:38
- JEWETT DL, ROMANO MN (1972) Neonatal development of auditory system potentials averaged from the scalp of rat and cat. *Brain Res* 36:101–115
- JOHNSON SL, FRANZ C, KNIPPER M, MARCOTTI W (2009) Functional maturation of the exocytotic machinery at gerbil hair cell ribbon synapses. *J Physiol* 587:1715–1726
- JOHNSON SL, ECKRICH T, KUHN S, ZAMPINI V, FRANZ C, RANATUNGA KM, ROBERTS TP, MASETTO S, KNIPPER M, KROS CJ, MARCOTTI W (2011) Position-dependent patterning of spontaneous action potentials in immature cochlear inner hair cells. *Nat Neurosci* 14:711–717
- JOHNSON SL, KUHN S, FRANZ C, INGHAM N, FURNESS DN, KNIPPER M, STEEL KP, ADELMAN JP, HOLLEY MC, MARCOTTI W. (2013A). Presynaptic maturation in auditory hair cells requires a critical period of sensory-independent spiking activity. doi: [10.1073/pnas.1219578110](https://doi.org/10.1073/pnas.1219578110) /-DCSupplemental.www.pnas.org/cgi/doi/10.1073/pnas.1219578110
- JOHNSON SL, WEDEMAYER C, VETTER DE, ADACHI R, HOLLEY MC, BELE A. (2013B). Cholinergic efferent synaptic transmission regulates the maturation of auditory hair cell ribbon synapses
- KANTARDZHIEVA A, LIBERMAN MC, SEWELL WF (2013) Quantitative analysis of ribbons, vesicles, and cisterns at the cat inner hair cell synapse: correlations with spontaneous rate. *J Comp Neurol* 521:3260–3271
- KHIMICH D, NOUVIAN R, PUJOL RR, TOM DIECK S, EGNER A, GUNDELFINGER ED, MOSER T, NOUBLAN R (2005) Hair cell synaptic ribbons are essential for synchronous auditory signalling. *Nature* 434:889–894
- KIM KX, RUTHERFORD MA (2016) Maturation of NaV and KV channel topographies in the auditory nerve spike initiator before and after developmental onset of hearing function. *J Neurosci* 36:2111–2118
- KROS CJ (1998) Expression of a potassium current in inner hair cells during development of hearing in mice. *Nature* 394:281–284
- LIBERMAN MC (1982) Single-neuron labeling in the cat auditory nerve. *Science* 216(80):1239–1241
- LIBERMAN LD, WANG H, LIBERMAN MC (2011) Opposing gradients of ribbon size and AMPA receptor expression underlie sensitivity differences among cochlear-nerve/hair-cell synapses. *J Neurosci* 31:801–808
- LIBERMAN LD, LIBERMAN MC (2016) Postnatal maturation of auditory-nerve heterogeneity, as seen in spatial gradients of synapse morphology in the inner hair cell area. *Hear Res* 339: 12–22
- LIU Q, DAVIS RL (2007) Regional specification of threshold sensitivity and response time in CBA/CaJ mouse spiral ganglion neurons. *J Neurophysiol* 98:2215–2222
- LIU W, DAVIS RL (2014) Calretinin and calbindin distribution patterns specify subpopulations of type I and type II spiral ganglion neurons in postnatal murine cochlea. *J Comp Neurol* 522:2299–2318
- MAKEINER S, LUO F, TAN A, SCHMITZ F, SÜDHOF TC. (2016). How to make a synaptic ribbon: RIBEYE deletion abolishes ribbons in retinal synapses and disrupts neurotransmitter release. doi: [10.15252/embj.201592701](https://doi.org/10.15252/embj.201592701)
- MEHTA B, SNELLMAN J, CHEN S, LI W, ZENISEK D (2013) Synaptic ribbons influence the size and frequency of miniature-like evoked postsynaptic currents. *Neuron* 77:516–527
- MERCHAN-PEREZ A, LIBERMAN MC (1996) Ultrastructural differences among afferent synapses on cochlear hair cells: correlations with spontaneous discharge rate. *J Comp Neurol* 371:208–221
- NAGIEL A, PATEL SH, ANDOR-ARDÓ D, HUDSPETH AJ (2009) Activity-independent specification of synaptic targets in the posterior lateral line of the larval zebrafish. *Proc Natl Acad Sci U S A* 106:21948–21953
- OHN T-L, RUTHERFORD MA, JING Z, JUNG S, DUQUE-AFONSO CJ, HOCH G, PICHER MM, SCHARINGER A, STRENZKE N, MOSER T (2016) Hair cells use active zones with different voltage dependence of Ca²⁺ influx to decompose sounds into complementary neural codes. *Proc Natl Acad Sci*. doi:[10.1073/pnas.1605737113](https://doi.org/10.1073/pnas.1605737113)
- ROTH B, BRUNS V (1992) Postnatal development of the rat organ of Corti. *Anat Embryol (Berl)* 185:571–581
- RUTHERFORD MA (2015) Resolving the structure of inner ear ribbon synapses with STED microscopy. *Synapse* 69:242–255
- RUTHERFORD MA, CHAPOCHNIKOV NM, MOSER T (2012) Spike encoding of neurotransmitter release timing by spiral ganglion neurons of the cochlea. *J Neurosci* 32:4773–4789
- SCHMITZ F, KÖNIGSTORFER A, SÜDHOF T (2000) RIBEYE, a component of synaptic ribbons: a protein's journey through evolution provides insight into synaptic ribbon function. *Neuron* 28:857–872
- SENDIN G, BULANKINA AV, RIEDEL D, MOSER T (2007) Maturation of ribbon synapses in hair cells is driven by thyroid hormone. *J Neurosci* 27:3163–3173
- SHEETS L, TRAPANI JG, MO W, OBHOLZER N, NICOLSON T (2011) RIBEYE is required for presynaptic Ca(V)1.3a channel localization and afferent innervation of sensory hair cells. *Development* 138:1309–1319
- SHEETS L, KINDT KS, NICOLSON T (2012) Presynaptic CaV1.3 channels regulate synaptic ribbon size and are required for synaptic maintenance in sensory hair cells. *J Neurosci* 32:17273–17286
- SIEGEL JH (1992) Spontaneous synaptic potentials from afferent terminals in the guinea pig cochlea. *Most* 59:85–92
- SIMMONS DD (2002) Development of the inner ear efferent system across vertebrate species. *J Neurobiol* 53:228–250
- SIMMONS DD, MANSION-GIESEKE L, HENDRIX TW, MCCARTER S (1990) Reconstructions of efferent fibers in the postnatal hamster cochlea. *Hear Res* 49:127–139
- SIMMONS DD, MANSORF NB, KIM JH (1996) Olivocochlear innervation of inner and outer hair cells during postnatal maturation: evidence for a waiting period. *J Comp Neurol* 370:551–562
- SOBKOWICZ HM, ROSE JE, SCOTT GE, SLAPNICK SM (1982) Ribbon synapses in the developing intact and cultured organ of Corti in the mouse. *J Neurosci* 2:942–957
- TABERNER A (2005) Response properties of single auditory nerve fibers in the mouse. *J Neurophysiol*. doi:[10.1152/jn.00574.2004](https://doi.org/10.1152/jn.00574.2004)
- TRITSCH NX, BERGLES DE (2010) Developmental regulation of spontaneous activity in the mammalian cochlea. *J Neurosci* 30:1539–1550
- WAGUESPACK J, SALLES FT, KACHAR B, RICCI AJ (2007) Stepwise morphological and functional maturation of mechanotransduction in rat outer hair cells. *J Neurosci* 27:13890–13902
- WALSH EJ, MCGEE J (1987) Postnatal development of auditory nerve and cochlear nucleus neuronal responses in kittens. *Hear Res* 28:97–116
- WONG AB, RUTHERFORD MA, GABRIELAITIS M, PANGRSIC T, GÖTTERT F, FRANK T, MICHANSKI S, HELL S, WOLF F, WICHMANN C, MOSER T (2014) Developmental refinement of hair cell synapses tightens the coupling of Ca²⁺ influx to exocytosis. *EMBO J* 33:247–264
- YI E, ROUX I, GLOWATZKI E (2010) Dendritic HCN channels shape excitatory postsynaptic potentials at the inner hair cell afferent synapse in the mammalian cochlea. *J Neurophysiol* 103:2532–2543
- YIN Y, LIBERMAN LD, MAISON SF, LIBERMAN MC (2014) Olivocochlear innervation maintains the normal modiolar-pillar and habenular-cuticular gradients in cochlear synaptic morphology. *JARO - J Assoc Res Otolaryngol* 15:571–583