Developmental Acquisition of a Rapid Calcium-Regulated Vesicle Supply Allows Sustained High Rates of Exocytosis in Auditory Hair Cells

Snezana Levic*, Yohan Bouleau, Didier Dulon*
Equipe Neurophysiologie de la Synapse Auditive, Unité Mixte de Recherche, Inserm US87 et Université Victor Segalen, Institut des Neurosciences de Bordeaux, Centre Hospitalier Universitaire Pellegrin, Bordeaux, France

Abstract
Auditory hair cells (HCs) have the remarkable property to indefinitely sustain high rates of synaptic vesicle release during ongoing sound stimulation. The mechanisms of vesicle supply that allow such indefatigable exocytosis at the ribbon active zone remain largely unknown. To address this issue, we characterized the kinetics of vesicle recruitment and release in developing chick auditory HCs. Experiments were done using the intact chick basilar papilla from E10 (embryonic day 10) to P2 (two days post-hatch) by monitoring changes in membrane capacitance and Ca\(^{2+}\) currents during various voltage stimulations. Compared to immature pre-hearing HCs (E10-E12), mature post-hearing HCs (E18-P2) can steadily mobilize a larger readily releasable pool (RRP) of vesicles with faster kinetics and higher Ca\(^{2+}\) efficiency. As assessed by varying the inter-pulse interval of a 100 ms paired-pulse depolarization protocol, the kinetics of RRP replenishment were found much faster in mature HCs. Unlike mature HCs, exocytosis in immature HCs showed large depression during repetitive stimulations. Remarkably, when the intracellular concentration of EGTA was raised from 0.5 to 2 mM, the paired-pulse depression level remained unchanged in immature HCs but was drastically increased in mature HCs, indicating that the Ca\(^{2+}\) sensitivity of the vesicle replenishment process increases during maturation. Concomitantly, the immunoreactivity of the calcium sensor otoferlin and the number of ribbons at the HC plasma membrane largely increased, reaching a maximum level at E18-P2. Our results suggest that the efficient Ca\(^{2+}\)-dependent vesicle release and supply in mature HCs essentially rely on the concomitant engagement of synaptic ribbons and otoferlin at the plasma membrane.

Introduction

The ribbon synapse of cochlear hair cells (HCs) encodes sound information by tightly controlling the number (discharge rate) and precise timing (temporal coding) of postsynaptic spikes. Remarkably, this synapse can drive postsynaptic auditory nerve fibers at extremely high instantaneous discharge rates (over several thousand spikes/s at stimulus onset) and, after rapid adaptation, can support sustained discharge rates over several hundred spikes/s during ongoing sound stimulation [1], [2]. To sustain such high rates of synaptic exocytosis, auditory HC must have efficient mechanisms to rapidly and constantly replenish the pool of synaptic vesicles. While it is well established that the rate of vesicle fusion is tightly controlled by Ca\(^{2+}\) ions flowing through nearby voltage-gated Ca\(^{2+}\) channels [3], little is known about the mechanisms regulating the kinetics of vesicle supply at the ribbon active zone. Recently, it was hypothesized that otoferlin, a multi-C2 Ca\(^{2+}\) sensor that directly regulates SNARE-membrane fusion in vitro [4] and is required for HC vesicle exocytosis [5], also controls the supply of synaptic vesicles at the active zones [6]. Surprisingly, during their early developmental period, immature auditory HCs transiently express several Ca\(^{2+}\)-dependent synaptic-totamins and do not require otoferlin to control phasic transmitter release driven by spontaneous action potentials [7]. With cochlear maturation, the Ca\(^{2+}\) efficiency and kinetics of exocytosis in HCs largely increase [8], [9], [10], as well as the expression level of otoferlin in HCs [5], [7]. However, the precise mechanisms of how otoferlin is engaged to produce fast vesicle supply and release in developing HCs remains to be elucidated.

Notably, the first sound-evoked responses of immature auditory nerve fibers in the developing cochlea display high thresholds of rhythmic bursting activity and are unable to maintain a sustained steady-state response to long duration tone bursts [11]. The present study tests the hypothesis that neurotransmitter availability is an important factor that limits sustained postsynaptic firing activity in developing auditory afferent neurons. By studying when the ability to produce sustained high rate of exocytosis is acquired by HCs during development, we attempt to gain insights into the mechanisms of synaptic vesicle replenishment. In chick embryo, the first indication of sound-evoked electrical responses from the inner ear have been reported from the 11th day of incubation (E11) [12], [13], [14]. The auditory thresholds then show continuous maturation between E15 and the first post-hatching day (P1) to attain adult values. In the present study, we took...
advantage of the slow maturation of the auditory chick basilar papilla to characterize the progressive changes occurring in exocytosis and vesicle supply at the HC ribbon synapse.

**Materials and Methods**

**Preparation of semi-intact chicken basilar papilla**

The present investigation was performed in accordance with the guidelines of the Animal Care and Use Committee of the European Communities Council Directive of November 24th, 1986 (86/609/EEC) and the University of Bordeaux (ethics committee: Direction Régionale de l’Alimentation, de l’Agriculture et de la Forêt d’Aquitaine [DRAAF Aquitaine]) permit number B 33075, approved for pClamp10 (Axon Instruments) and Origin7.0 (Microcal Software). Pooled data were presented as mean ± SD. Significant difference between groups of cells or between different embryonic stages of development was evaluated using a two-tailed Student’s t test; p values are presented in the text and figure to indicate statistical significance. Time constants (\(\tau\)) were obtained from fits using Origin software. Time constants were obtained by fitting multiple exponential equations to the activation decay of the current. The equation was of the form:

\[
I = I_0 + A_1 \cdot \exp\left(-t/\tau_1\right) + A_2 \cdot \exp\left(-t/\tau_2\right) + A_n \cdot \exp\left(-t/\tau_n\right).
\]

Where \(I_0\) is the initial current magnitude, \(\tau_1, \tau_2, \ldots, \tau_n\) are the time constants, and \(A_1, A_2, \ldots, A_n\) are the proportionality constants. Synaptic transfer functions relating Ca\(^{2+}\) current (\(I_{Ca}\)) and Ca\(_m\), or \(Q_{Ca}\) and \(Cm\) were calculated using an integral of total \(I_{Ca}\) including the tail currents. The data was fitted using first-order power functions:

\[
\Delta C_m = s[I_{Ca}]^N \text{ or } \Delta C_m = s[Q_{Ca}]^N
\]

where \(s\) = slope factor (fF/pA or fF/pC), and \(N\) = power index.

The % RRP refilling was calculated as:

\[
%\text{RRPrefilling} = \left(\frac{\Delta C_m - \Delta C_{m,\text{test}}}{\Delta C_m}\right) \times 100
\]

where \(\Delta C_m = \Delta C_{m,\text{meas}}\) measured using the first, control pulse, and \(\Delta C_{m,\text{test}} = \Delta C_{m,\text{measured using test pulse}}\). The % of \(I_{\text{recovered}}\) was calculated as:

\[
%I_{\text{recovered}} = \left(\frac{I_{Ca,\text{control}} - I_{Ca,\text{test}}}{I_{Ca,\text{control}}}\right) \times 100
\]

Where \(I_{Ca,\text{control}} = I_{Ca}\) measured using the first, control pulse, and \(I_{Ca,\text{test}} = I_{Ca,\text{test}}\) measured using test pulse.

**Results**

**Kinetics and Ca\(^{2+}\)-efficiency of RRP exocytosis increase with cochlear maturation**

The efficiency of Ca\(^{2+}\)-evoked exocytosis was characterized at four developmental periods of cochlear synaptogenesis: embryonic
stages (in ovo) E10–11, E12–14, E16–18 and 2 days post-hatching P2. The first embryonic period, E10–11, corresponds to an early stage of synaptogenesis when the first presynaptic specializations (synaptic bodies or ribbons) can be detected in HCs [18] and when the afferent fibers first contact their base [19]. At stage E11–E14, low frequency hearing starts in the chick embryo [12], [13], [14]. Stage E16–E18 (5-3 days before hatching) corresponds to the final step of synaptogenesis and HC maturation. Finally, P2 corresponds to nearly adult hearing values [20].

At all developmental stages from E10 to P2, rapidly activating inward current (ICa) and a concomitant increase in membrane capacitance ($\Delta C_m$) was recorded when HCs were voltage-stepped from -90 mV to varying depolarized potentials (Fig. 1A–1B). The voltage-activation curve of $\Delta C_m$ displayed a bell shape that followed the $I_{Ca}$ activation curve (maximum amplitude near -10 mV), a behavior consistent with $\Delta C_m$ being activated consecutive to $Ca^{2+}$ influx. Indeed, complete blockage of ICa by 250 $\mu$M CdCl$_2$ eliminated $\Delta C_m$ (data not shown), confirming that $\Delta C_m$ is sensitive to $Ca^{2+}$ entry via VGCC, in agreement with [21].

For a 100 ms-depolarization, which is considered to entirely release the readily releasable pool (RRP) [22], the amplitude of $\Delta C_m$ responses increased with HC maturation (Fig 1A–B). In low frequency apical HCs, $\Delta C_m$ responses were as follows: (in fF at -10 mV): E10, 10±2 (n = 10); E12, 29±6 (n = 13); E16, 37±7 (n = 11); and P2, 45±6 (n = 9). High frequency HCs recorded at the base of the basilar papilla were also found to undergo a similar increase in $\Delta C_m$ responses with maturation (Fig. 1A–B and Table 1). Notably, at embryonic stages earlier than E10, while activating significant $I_{Ca}$ (17.5±3.1 pA at -10 mV, n = 7, E7–E8), 100-ms voltage-step depolarization did not produce significant $\Delta C_m$ responses in all HCs tested (below background level of 3.8±1.3 fF; data not shown).

Figure 1. $Ca^{2+}$ efficiency of RRP exocytosis increases with maturation. (A) Examples of $I_{Ca}$ and $\Delta C_m$ recordings following a 100 ms-voltage step from holding potential of -90 mV to -10 mV at E10, E12, E16, and P2 basal HCs. (B) Voltage-dependence of $I_{Ca}$ and $\Delta C_m$ recorded in basal HCs at E10 (n = 6), E12 (n = 9), E16 (n = 9) and P2 (n = 7); $I_{Ca}$ was measured at its peak value during the voltage pulse shown in A. To limit depression, each consecutive voltage-step was separated by a 30 s recovery period. (C) Synaptic transfer functions relating $Q_{Ca}$, (charge integral of $Ca^{2+}$ current) and $\Delta C_m$ in basal HCs. Data points were fitted using first order power function with $\Delta C_m = s[I_{Ca}]^N$, where $s$ = slope factor ($Ca^{2+}$ efficiency; fF/pC) and $N$ = power index or degree of co-operativity. Values of $s$ and $N$ are reported in Table 1. (D) Comparative $Ca^{2+}$ efficiency ($\Delta C_m/Q_{Ca}$) in apical (E10, n = 10; E12, n = 13; E16, n = 11; and P2, n = 9) and basal HCs (n as in B) as a function of developmental age. (E) Comparative cooperative (power) index N from power fit of data in D as a function of age in developing apical and basal HCs.
Table 1. Main characteristics of Ca²⁺ dependence of exocytosis in developing chick HCs (LF = low frequency and HF = high frequency).

| Exocytosis | E10       | E12       | E16       | P2        |
|------------|-----------|-----------|-----------|-----------|
| Ca²⁺ dependence (N) |           |           |           |           |
| Apical (LF) | 1.58±0.14 | 1.44±0.34 | 1.14±0.12 | 0.85±0.07 |
| Basal (HF)  | 1.54±0.21 | 1.61±0.38 | 1.56±0.27 | 1.05±0.11 |
| Ca²⁺ efficiency (IF/pC) |           |           |           |           |
| Apical (LF) | 0.58±0.13 | 0.49±0.27 | 1.6±0.5  | 6.35±0.97 |
| Basal (HF)  | 1.14±0.4  | 0.45±0.35 | 2.92±1.2 | 6.89±0.63 |
| RRP release rate (vesicles/s) |           |           |           |           |
| Apical (LF) | 3158±698  | 10198±2245 | 18318±3253 | 30540±4122 |
| Basal (HF)  | 3720±1126 | 11393±1828 | 22015±4623 | 38687±4460 |
| Total vesicles in RRP (100 ms) |           |           |           |           |
| Apical (LF) | 281±62    | 764±168   | 989±176   | 1221±164  |
| Basal (HF)  | 316±96    | 827±133   | 1056±221  | 1354±153  |

N indicates power (cooperative) index.
doi:10.1371/journal.pone.0025714.t001

The synaptic transfer function relating ΔC_
Ca
 as a function of charge entry (Q_
Ca
, as time integral of I_
Ca
) was compared at different developmental stages by stepping the cells to various potentials from -60 to -10 mV for a constant 100 ms duration (Fig. 1C). With maturation, data points both in apical and basal HCs were fitted by first-order power functions with decreasing power index (N, cooperative index) and increasing slope factors (Ca²⁺ sensitivity, IF/pC) (Fig. 1D-E; table 1). These results indicated that, similarly to mouse cochlear HCs [8], [9], the kinetics of RRP release were largely increased with secondary, slowly releasable pool (SRP) as previously described [9], [23]. Maturation of the chick HC synapse is associated with a better coupling between Ca²⁺ influx and vesicular release.

Changes in release rate were then compared by stepping HCs to constant potential (from -90 to -10 mV) for different durations from 20 to 3000 ms (Fig. 2). Data points were best fitted by two exponential functions that likely described a fast release of a readily releasable pool (RRP) of vesicles (up to 100 ms) and a secondary, slowly releasable pool (SRP) as previously described [9], [21], [23]. Kinetics of RRP release largely increased with maturation from E10 to P2, with respective time constants of RRP recovery below 200 ms because capacitance measurements are obtained in mature chick HCs are in agreement with previous findings [22] and suggest that the supply of vesicle to the RRP is poorly Ca²⁺-sensitive (Fig. 4A, 4C, 4D). By contrast, reduced intracellular Ca²⁺ availability with high EGTA largely increased paired-pulse depression in immature HCs [e.g. at P2, 95±2% (n = 4) p<0.001; table 2]. We did not refine the kinetics of paired-pulse recovery below 200 ms because capacitance measurements are altered by Ca²⁺ tail currents below this time frame.

Next, we compared the rate of vesicle supply to the RRP when using an intracellular recording solution containing 2 mM EGTA instead of 0.5 mM (Fig. 4). Surprisingly, at E12 using either 0.5 mM or 2 mM EGTA, the RRP showed a similar level of paired-pulse depression, indicating that the refilling rate was poorly Ca²⁺-sensitive (Fig. 4A, 4C, 4D). By contrast, reduced intracellular Ca²⁺ availability with high EGTA largely increased paired-pulse depression in mature HCs [e.g. at P2, (mM EGTA, % RRP recovery 200 ms after the first 100 ms-pulse): 0.5, 95±2% (n = 4); 2, 10±2% (n = 4) p<0.001; Fig. 4B, 4C, 4D]. These results obtained in mature chick HCs are in agreement with previous findings [22] and suggest that the supply of vesicle to the RRP is Ca²⁺-sensitive. The novelty of our findings is that this vesicle supply process in immature HCs and mature HCs shows a different Ca²⁺ sensitivity.

The efficiency of vesicular recruitment increases during development and allows sustained release.

The RRP replenishment was also studied by stimulating the hair cells with a 100 ms paired-pulse depolarization protocol (Fig. 3). In both basal and apical immature HCs, exocytosis showed marked paired-pulse depression that decreased with maturation from 25% in E12 to 5% in P2 HCs (Fig. 3A-3B). When varying the interpulse interval, approximately 95% of the RRP was restored within ~6 s at E12, and ~0.7 s at E16 (e.g. time constants (s) E12: best fit with dual exponential function with τ₁ = 0.8±0.1 and τ₂ = 6.1±1.4 (n = 5); E16: best fit with a single exponential function τ = 0.7±0.1 (n = 5); p<0.05; Fig. 3C). Notably, marked paired-pulse depression or inactivation was also observed for I_
Ca
 in immature E12 HCs as compared to mature P2 HCs. The kinetics of RRP recovery in E12 HCs paralleled the time course of I_
Ca
 recovery (Fig. 3D), indicating that ΔC_
Ca
 paired-pulse depression was mainly due to I_
Ca
 inactivation in immature HCs. By contrast, mature HCs reconstituted ~95% of the RRP within less than 200 ms and showed almost no I_
Ca
 inactivation (e.g. P2: 95±2% (n = 4) p<0.001; table 2). We did not refine the kinetics of paired-pulse recovery below 200 ms because capacitance measurements are altered by Ca²⁺ tail currents below this time frame.

As a function of maturation and allows sustained release.
Cumulative $\Delta C_m$ responses showed marked depression in immature developing HCs, as indicated by a progressive decrease in $\Delta C_m$ responses during the repetitive stimuli: In basal high frequency HCs, $\Delta C_m$ decreased from a mean of 27 ± 4 fF after the first stimulus to 10 ± 3 fF after the 20th one at E12 (n = 5; $p < 0.001$; Fig. 5G). Similar depression was observed in E12 low frequency HCs (Fig. 5D). In these immature HCs, the Ca$^{2+}$ efficiency of vesicle release ($\Delta C_m/Q_Ca$) from RRP remained constant during the repetitive stimulations (Fig. 5E, 5H), indicating that the depression of exocytosis mainly arose from the marked inactivation of the Ca$^{2+}$ current (Fig. 5A). By contrast, mature P2 HCs from base or apex showed no depression of the RRP, as indicated by a near linear increase in cumulative $\Delta C_m$ (Fig. 5B–5F). Mature P2 HCs showed constant high Ca$^{2+}$ efficiency in exocytosis (Fig. 5E, 5H) and absence of ICa inactivation (Fig. 5B).

Otoferlin expression and number of synaptic ribbons per HC increase with maturation

Several factors could explain the increase in kinetics and Ca$^{2+}$ efficiency of exocytosis in HCs during development: a reduction in the distance between the Ca$^{2+}$ channels and the sites of release at the active zone; a change in the affinity of the Ca$^{2+}$ sensor that controls membrane fusion; or a reduction in the diffusion barrier of Ca$^{2+}$ ions at the site of release. We found that the average number of ribbons per HC largely increased with development from $0.3 \pm 0.5$ (n = 50) at E8, $2.1 \pm 1.6$ (n = 50) at E12, $3.1 \pm 1.4$ (n = 50) at E16 and $9.3 \pm 2.2$ (n = 50) at P2 (Fig. 6A and table 2). These results indicate a positive correlation between Ca$^{2+}$ efficiency in exocytosis and the number of synaptic ribbons in HCs. Our observations are in agreement with the recent finding that the synaptic ribbons contribute largely to synaptic neurotransmission by facilitating high rates of exocytosis, while their absence significantly compromise the temporal resolving power of the auditory system [25], [26].

We then used the recently characterized monoclonal antibody HCS-1 [17] to explore the expression of the calcium sensor otoferlin during development of chick basilar papillae. Otoferlin was weakly expressed in HCs at embryonic stages earlier than E10 and then increased with development to reach a maximum level at E18-P2 (Fig 6B). At these late stages of development, HCS-1 immunolabeling was largely distributed at the plasma membrane from the apical part of the HCs (below the cuticular plate) to the lower end of the HCs (synaptic area). It is to be mentioned that, in absence of a true control as in mouse knock out for the otoferlin gene [5], [7], we cannot ascertain that the HCS-1 labeling is entirely specific. However, it is to be noted that both the plasma membrane labeling and the developmental increase of HCS-1 labeling matched very well the recent results obtained in mouse HCs [7].

Discussion

This report characterizes the functional changes occurring during progressive maturation of the HC synaptic machinery in a precocial post-hearing vertebrate, the chick, where sound-evoked cochlear nuclei activity can be measured as early as E11 in ovo [12], [13]. Concomitantly to an increased expression of ribbons and otoferlin, exocytosis of chick HCs progressively displayed faster kinetics and higher Ca$^{2+}$ efficiency with maturation. Similar changes have been shown in HCs of pre-hearing animals such as mouse and gerbil [9], [23]. Our study demonstrates for the first time a positive correlation between Ca$^{2+}$ efficiency in exocytosis and the number of synaptic ribbons in HCs.
time that vesicle supply and RRP release undergo a parallel maturation to allow mature HCs to sustain high rates of exocytosis. In addition, we show that vesicle recruitment is highly Ca$^{2+}$-dependent in mature chick HCs, in agreement with previous findings [22]. Notably, a constant vesicle trafficking from a reserve pool has also been recently proposed to be Ca$^{2+}$-dependent in turtle auditory HCs [27].

Remarkably, immature chick HCs displayed significant depression in exocytosis during repetitive brief stimuli or paired-pulse stimulation, while mature HCs showed little RRP depression. This exocytotic depression in immature HCs is likely due in part to the rapid inactivating property of the Ca$^{2+}$ current at this developmental stage. Indeed, the Ca$^{2+}$ current and the RRP showed similar kinetics of recovery during paired-pulse stimulations. Notably, while the Ca$^{2+}$ current of mature chick HCs is mainly driven by non-inactivating dihydropyridine-sensitive L-type Ca$^{2+}$ channels [28], [29], immature chick HCs (in addition to L-type channels) transiently express fast inactivating T-type Ca$^{2+}$ channels, [16] and unpublished data. Furthermore, L-type Ca$^{2+}$ currents of immature HCs display strong calmodulin-mediated calcium-dependent inactivation [30]. Therefore, Ca$^{2+}$ current inactivation leading to RRP depression could partially explain the transient rhythmic temporal discharge pattern of the auditory nerve fibers observed in young kittens [11].

Figure 3. Kinetics of RRP replenishment increase during development. (A) Recording examples of $I_{Ca}$ and D$C_m$ responses during a paired-pulse protocol (two consecutive 100 ms steps from -90 mV to -10 mV separated by 200 ms) at E12 and P2 in apical and basal tall HCs. (B) Comparative paired-pulse recovery (% of first response) at E10 (n = 7), E12 (n = 8), E16 (n = 7) and P2 (n = 4). Recordings were made with an intracellular Ca$^{2+}$ buffer of 0.5 mM EGTA. Note a larger depression in immature pre-hearing HCs E10–E13 as compared to P2 mature HCs. Asterisks indicate a statistical difference with $p<0.01$. (C) Comparative kinetics of RRP recovery in E12 and E16 apical HCs when varying the inter-pulse time in the paired-pulse protocol. In immature E12 HCs, data points were best fitted with two exponentials with time constants of 800 ms and 6 s (n = 5). In E16 HCs (n = 5), data points were best fitted with a single exponential with time constant of 680 ms. (D) During the paired-pulse protocol, in E12 apical HCs, RRP and $I_{Ca}$ showed similar depression or slow kinetics of recovery.

doi:10.1371/journal.pone.0025714.g003

Table 2. Vesicle replenishment characteristics in apical HCs.

|                         | E10       | E12       | E16       | P2        |
|-------------------------|-----------|-----------|-----------|-----------|
| **Paired pulse depression 100ms pulse - 200ms interval** |           |           |           |           |
| 0.5 mM EGTA             | not tested| 25±9%     | 30±5%     | 5±2%      |
| 2 mM EGTA               | not tested| not tested| 90±2%     |           |
| **RRP replenishment time constant** |           |           |           |           |
| $t_1$                   | 850ms     | 800 ms    | 680 ms    | <200ms    |
| $t_2$                   | 7s        | 6s        | -         |           |
| **Number of ribbons per cell** | 0.3±0.5  | 2.1±1.6   | 3.1±1.4   | 9.3±2.2   |

doi:10.1371/journal.pone.0025714.t002
and in chicken embryos [14], [31]. These immature animals show high threshold low frequency hearing and are unable to maintain a sustained steady-state response to long duration tone bursts [32].

In agreement with [22], we found that RRP replenishment, but not release, was diminished by using 2 mM EGTA instead of 0.5 mM in mature chick HCs. These results suggest that the release sites are less than 200 nm from Ca\(^{2+}\) entry, while the reloading sites extend farther than 200 nm. Similar Ca\(^{2+}\) regulation of vesicle replenishment has been shown at the cone ribbon synapses of the retina [33]. In HCs of the amphibian papilla, recovery from paired-pulse depression has recently shown to be ultrafast and also dependent on Ca\(^{2+}\) [34]. The most intriguing result of our study was the observation that the RRP recovery of immature HCs, unlike mature chick HCs, was not sensitive to 2 mM intracellular EGTA. This may reflect differences in the distance of the stock of vesicular supply from the release sites and Ca\(^{2+}\) entry during development. A larger and wider extrasynaptic distribution of Ca\(^{2+}\) channels at the early stage of development, as shown in immature mouse HCs [35], could place Ca\(^{2+}\) entry closer to the refilling machinery (reserve pool of vesicles) in immature HCs and in turn make vesicle replenishment

---

**Figure 4. Rate of vesicle replenishment becomes highly sensitive to intracellular EGTA with maturation.**

A) Two paired-pulse recordings of \(I_{Ca}\) and \(\Delta C_m\) in response to two consecutive 100 ms depolarizing steps (as in Fig 3) separated by either 200 ms or 1 s in an E12 apical tall HC using 2 mM intracellular EGTA. (B) Two paired-pulse recordings of \(I_{Ca}\) and \(\Delta C_m\) as in A but in mature P2 apical HCs. Note the strong depression in \(\Delta C_m\) after the 2\(^{nd}\) pulse. (C) Comparative RRP recovery after 200 ms inter-pulse using 0.5 or 2 mM intracellular EGTA in E12 (\(n = 5\)) and P2 (\(n = 5\)) apical HCs. Asterisk indicates statistical significance with \(p < 0.01\). (D) Comparative kinetics of RRP recovery using 0.5 or 2 mM intracellular EGTA in mature P2 (\(n = 5\)) or immature E12 (\(n = 5\)) apical HCs.

doi:10.1371/journal.pone.0025714.g004
less sensitive to EGTA. Notably, we found a positive correlation between the increasing number of ribbons with maturation and the efficiency of vesicle supply and release. Our results obtained in developing HCs are in good agreement with those obtained in a recent study using transgenic mice lacking the presynaptic scaffold protein bassoon, an essential element to dock the ribbon to the active zone [26]. The latter study concluded that the ribbon is essential for organizing Ca\textsuperscript{2+} channels and vesicles in the synaptic active zone in order to promote efficient vesicle replenishment.

In addition to an increased number of ribbons during maturation, the organization of a different Ca\textsuperscript{2+}-dependent vesicle supply may also progressively take place in mature HCs. Otoferlin, which is considered to be a high affinity Ca\textsuperscript{2+} sensor that directly triggers SNARE-membrane fusion in vitro [4] and regulates Ca\textsuperscript{2+}-evoked membrane fusion at the ribbon synapse of both cochlear [5] and vestibular HCs [36], could also regulate a Ca\textsuperscript{2+}-dependent vesicle supply at a large distance from Ca\textsuperscript{2+} entry. Indeed, we found that the expression of otoferlin increases at the right period of cochlear maturation when vesicle replenishment becomes efficient, suggesting a progressive engagement of this Ca\textsuperscript{2+} sensor in the ribbon active zone. By progressively replacing other Ca\textsuperscript{2+} sensors such as synaptotagmins during development [7], otoferlin may facilitate vesicle supply and release at the mature HC ribbon synapse.

Contrary to mature auditory HCs, the ribbon synapses of the retina do not express otoferlin [17], [37] and display pronounced paired-pulse depression that is attributable to a limiting slow replenishment of vesicles [38], [39]. Indeed, RRP recovery in retinal bipolar neurons displays rather slow kinetics (τ ~ 4 to 8 s) spanning the range of what we found in immature HCs. Our present study, showing a concomitant developmental onset of a fast Ca\textsuperscript{2+}-sensitive vesicle supply and otoferlin expression, suggests that this multi-C2 protein may also act as a Ca\textsuperscript{2+} sensor for the recruitment of vesicles located far from the release sites and Ca\textsuperscript{2+} channels, and probably farther than 200 nm as suggested by its sensitivity to 2 mM EGTA. This hypothesis is also reinforced by the phenotype of the pachanga mouse model, which carries a missense mutation in the C2F domain of otoferlin, and where the

Figure 5. An highly efficient vesicle recruitment allows sustained release in mature HCs. A) Recording examples of Cm and I\textsubscript{Ca} from an E12 apical HC during a train of 100 ms pulses (from -90 mV to -10 mV), each separated by 200 ms. B) Recruitment example evoked in similar conditions in a P2 apical HC. C–D–E) Apical HCs: Comparative mean cumulative ∆Cm changes over number of stimuli in E12 (n = 5) and P2 (n = 4) HCs (C). Comparative mean ∆Cm changes over number of stimuli (D). Contrary to mature P2 HCs, E12 immature HCs cannot sustain constant exocytosis during the train of stimuli. The comparative Ca\textsuperscript{2+} efficiency (fF/pC) calculated after each stimulus is shown in E. (F, G, H) Summary data comparing cumulative ∆Cm changes and Ca\textsuperscript{2+} efficiency at E12 (n = 5), and P2 (n = 4) in basal HCs.

doi:10.1371/journal.pone.0025714.g005
replenishment process of synaptic vesicles is affected independently of RRP fusion [6].

Acknowledgments

We thank Maryline Beurg, Ebenezer Yamoah and Christine Petit for discussion.

References

1. Kiang N Y-S, Watanabe T, Thomas EG, Clark LF (1965) Discharge Patterns of Single Fibers in the Cat’s Auditory Nerve. Cambridge MA: M.I.T Press.
2. Furukawa T, Matsumura S (1978) Adaptive rundown of excitatory post-synaptic potentials at synapses between hair cells and eighth nerve fibres in the goldfish. J Physiol (Lond) 276: 193–209.
3. Fuchs PA, Glowatzki E, Moser T (2003) The afferent synapse of cochlear hair cells. Curr Opin Neurobiol 13: 452–458.
4. Johnson CP, Chapman ER (2010) Otoferlin is a calcium sensor that directly regulates SNARE-mediated membrane fusion. J Cell Biol 191: 187–197.
5. Roux I, Safieddine S, Norris D, Grati M, Simmler MC, et al. (2006) Otoferlin, defective in a human deafness form, is essential for exocytosis at the auditory ribbon synapse. Cell 20: 277–289.
6. Pangscic T, Lasarow L, Reuter K, Takago H, Schwander M, et al. (2010) Hearing requires otoferlin-dependent efficient replenishment of synaptic vesicles in hair cells. Nat Neurosci 13: 869–876.
7. Beurg M, Michalski N, Safieddine S, Boulan Y, Schneggenburger R, et al. (2010) Control of Exocytosis by Synaptotagmin and Otoferlin in Auditory Hair Cells. J Neurosci 30: 13281–13290.

Figure 6. CtBP2 protein (RIBEYE) and otoferlin expression in developing chick HC. A) Surface preparations of the chick basilar papilla at various developmental stages were labeled with CtBP2 (green) and phalloidin antibodies (blue). Confocal images are averaged over 5–9 Z-stack images of 0.4 μm each and taken from the nucleus area to the bottom of the cell. At right, the graphs indicate the corresponding distribution of the averaged number of ctBP2 spots (ribbons) per HCs from 5 basilar papilla in the low frequency region. Lower bar scale indicates10 μm. B) Confocal images showing otoferlin (red) expression at different developmental stages in the apical low frequency region of chick cochlea (E9, E12 and P2). Note the increasing expression of otoferlin at the HC plasma membrane with development.

doi:10.1371/journal.pone.0025714.g006

Author Contributions

Conceived and designed the experiments: SL, DD. Performed the experiments: SL, YB. Analyzed the data: SL, DD. Contributed reagents/materials/analysis tools: SL, YB, DD. Wrote the paper: SL, DD.
Beutner D, Moser T (2001) The Presynaptic Function of Mouse Cochlear Inner Hair Cells during Development of Hearing. J Neurosci 21: 4593-4599.

Johnson SL, Marcotti W, Kros CJ (2005) Increase in efficiency and reduction in Ca2+ dependence of exocytosis during development of mouse inner hair cells. J Physiol (Lond) 563: 177–191.

Brug M, Safieddine S, Roux I, Boucle Y, Petit C, et al. (2008) Calcium- and Otoferlin-Dependent Exocytosis by Immature Outer Hair Cells. J Neurosci 28: 1790–1803.

Walsh EJ, Romand R (1992) Functional development of the cochlea and the cochlear nerve, Chapter 6 pp 161-219, in Development of auditory and vestibular system 2, Romand (Ed). Elsevier Science Publishers BV.

Saunders JC, Coles RB, Richard Gates G (1973) The development of auditory evoked responses in the cochlea and cochlear nuclei of the chick. Brain Res 5: 59–74.

Rebillard G, Rubel EW (1981) Electrophysiological study of the maturation of auditory responses from the inner ear of the chick. Brain Research 229: 13–23.

Jones TA, Jones SM, Paggett KC (2006) Emergence of Hearing in the Chicken Embryo. J Neurophysiol 96: 129–141.

Hamburger V, Hamilton HL (1992) A series of normal stages in the development of the chick embryo. Developmental Dynamics 195: 231–272.

Levic S, Nie I, Tuteja D, Harvey M, Sokolowski BH, et al. (2007) Development and regeneration of hair cells share common functional features. Proc Natl Acad Sci USA 104: 19108–19113.

Goodyear R, Logan P, Christiansen J, Xia B, Korchagina J, et al. (2010) Identification of the Hair Cell Soma-1 Antigen, HCS-1, as Otoferlin. J Assoc Res Otolaryngol 11: 573–586.

Rebillard M, Pujol R (1983) Innervation of the chicken basilar papilla during its development. Acta Otolaryngol 96(5–6): 379–88.

Whitehead MC, Morest DK (1985) The development of innervation patterns in the avian cochlea. Neurosci 14: 255–276.

Johnson SL, Marcotti W, Kros CJ (2005) Increase in efficiency and reduction in Ca2+ dependence of exocytosis during development of mouse inner hair cells. J Physiol (Lond) 563: 177–191.

10. Beutner D, Moser T (2000) Kinetics of exocytosis and endocytosis at the cochlear inner hair cell afferent synapse of the mouse. Proc Natl Acad Sci USA 97: 883–888.

Buran SN, Neef A, Gundelfinger ED, Moser T, Liberman MC (2010) Onset Coding Is Degraded in Auditory Nerve Fibers from Mutant Mice Lacking Synaptic Ribbons. J Neurosci 30: 7387–7397.