Supplementary Material 6 Characterization of the biophysical properties of Ca^{2+} channels in IHCs during postnatal development

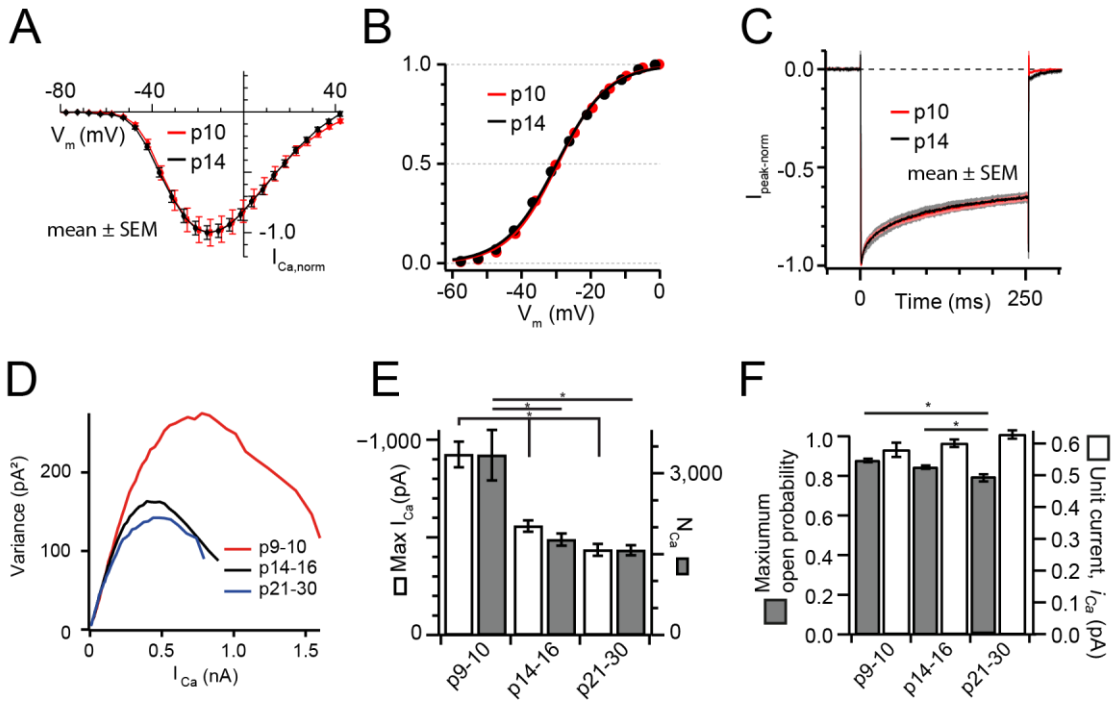

Figure S6. Biophysical properties of voltage-dependent whole-cell Ca^{2+} current (I_{Ca}) (A) Average steady-state I_{Ca}-V for p10 (red) and p14-16 (black) IHCs in 5 mM $[Ca^{2+}]_e$, normalized to peak I_{Ca} (for panels A and B: p10, n = 23 IHCs; p14-16, n = 31 IHCs). (b) Activation curve for I_{Ca}, obtained by dividing the I_{Ca}-V curve in (a) by a line-fit to the linear portion (-5 to +20 mV). Solid lines represent fitting of a Boltzmann function in the form of $1/(1+\exp((V_{half}-V)/slope)$. No significant difference was found in the half-activation potential (V_{half}; p10: -29.74 ± 0.35 mV; p14-16: -29.73 ± 0.36 mV,; Student's t-Test, p = 0.97), despite a subtle difference in their slope (p10: 7.10 ± 0.09 mV; p14-16: 7.39 ± 0.06 mV; Wilcoxon Rank Test, p = 0.0049). (C) Average inactivation of I_{Ca} during a 254 ms depolarization in 5 mM $[Ca]_e$ and highly buffered (10 mM $[EGTA]_i$) condition. No obvious difference was observed between the inactivation kinetics of the two age groups (6 repetitions per cell; p10, n = 7 IHCs; p14-16, n = 7 IHCs). (D) Grand averages of variance vs. mean relationships (p9-10: n = 13 IHCs, p14-16: n = 31 IHCs, p21-30: n = 27 IHCs) in nonstationary fluctuation analysis of Ca^{2+} tail-currents. $[Ca^{2+}]_e$ = 10 mM, $[BayK8644]_e$ =5µM. (E)

The decrease in whole cell Ca^{2+} current (I_{Ca}, empty bars; p9-10, n = 13 IHCs; p14-16, n = 31 IHCs; p21-30, n = 27 IHCs) was largely matched by the reduction in number of Ca^{2+}-channel estimated by fluctuation analysis (N_{Ca}, solid bars; p9-10, n = 16 IHCs; p14-16, n = 33 IHCs; p21-30, n = 26 IHCs) during postnatal development (F) There was no significant change in the estimated single channel current (holding potential: -68 mV; p9-10: 0.58 ± 0.02 pA, n = 13 IHCs; p14-16: 0.60 ± 0.01 pA, n = 31 IHCs; p21-30: 0.63 ± 0.01 pA, n = 27 IHCs; ANOVA, p = 0.103) and only a small reduction in the estimated maximal open probability measured (p9-10: 0.88 ± 0.01; p14-16: 0.84 ± 0.01; p21-30: 0.79 ± 0.02; Student's t-test, p9-10 vs p14-16, p = 0.27; p9-10 vs p20-30, $p < 0.001$; p14-16 vs p20-30, p = 0.006).

Comparing the estimation of microscopic channel properties by non-stationary fluctuation analysis and direct cell-attached single channel recordings

The main point of doing the fluctuation analysis was to seek confirmation of the hypothesis that the number of Ca^{2+} channels (N_{Ca}) declines around the onset of hearing. This was, indeed, observed and also agrees with previously published work based on single channel recordings[2]. However, we note that both approaches differed in their estimates of single channel current i_{Ca} and open probability (P_{open}), which we believe reflects technical differences that are discussed below.

Generally, fluctuation analysis tends to underestimate the i_{Ca} due to the limited bandwidth of recording (8.5 kHz), and this seems to be the case here, too. Our estimates of approximately -0.6 pA at -68 mV compare to approximately -1.0 pA measured in cell-attached at -68 mV after hearing onset[3]. For this reason we relied on the single channel recording estimates for i_{Ca} for our biophysical model.

We believe that likely methodological differences gave rise to the differences between the P_{open} estimates from single channel experiments (0.15 at -20 mV for immature IHCs[2] and 0.21 for mature IHCs[3] of the gerbil) and fluctuation analysis (around 0.8 at approximately +60 mV in estimates from mature IHCs [present study and refs. 4,5] and slightly higher (0.88) for immature IHCs). Using BayK8644 and strong depolarization we aim to maximize the open probability (greater than 0.5) as required for faithful estimation of the channel properties by binomial fitting. As stated in the main manuscript we aim to estimate the total number of Ca^{2+} channels and identify our open probability estimates as "maximal open probability" to make these points. While the single channel work by ref.

2,3 also used BayK8644, they used lower depolarizations and longer pulses (mostly 500 ms). We consider it likely that the lower depolarization level (via less activation) and potentially also Ca^{2+} dependent inactivation led to lower estimates of open probability. Since Ca^{2+} dependent inactivation is greater in immature than in mature IHCs[6] one would then expect lower open probability estimates in immature IHCs, which seems to be the case (see above). Further observations of ref. 2,3 fitting this hypothesis are that the bursts (mode 2 of gating) were preferentially observed at the beginning of the sweep and that inactivation is evident in the ensemble currents. For this reason in our biophysical model we relied on the P_{open} estimate of the fluctuation analysis. We used P_{open} of 0.4, which we derived from the measured 0.8 and divided it by the factor of 2 by which BayK8644 enhances the whole cell Ca^{2+} current at the peak Ca^{2+} current potential[5,7]. We note that this likely reflects an upper boundary, which would in the model work against a dominance of the nearest channel for controlling Ca^{2+} at a given vesicle (i.e. against Ca^{2+} nanodomain control, that we favor for the mature AZ).