SI Table 1.

Comparison of properties of T-type Ca^{2+} currents

Reference	Tissue	$[Ca^{2+}]_{ext}$, mM	IV_{peak}, mV	$V_{0.5act}$, mV	$V_{0.5inact}$, mV	$\tau_{deact\ 1,2}$, ms	$\tau_{decay1,2}$, ms	$\tau_{recovery}$, ms	Nickel-sensitive
This study	Chicken hair cells	5	-15	-38	-37	0.35 24	35 330	40 1,200	Yes
1	Olfactory	3	-40	-45	-66	-	-	-	Yes
2	Cone bipolar	10	-30	-36	-59	-	30	-	Yes
3	Myocytes	2	-30	-42	-68	1.3	13		Yes
4	Skeletal muscle	10	-15	-24	-41	-	16	224 5,160	Yes
5	Cochlear nucleus	2	-40	-59	-89	-	-	-	Yes

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SI Experimental Procedures

Isolation of the Chick Basilar Papilla. The present investigation was performed in accordance with the guidelines of the Animal Care and Use Committee of the University of California, Davis. This study includes chickens at different stages of embryonic development ranging from E6-E21 as well as post hatched chickens. Fertilized eggs were incubated at 37°C in a Marsh automatic incubator (Lyon Electric). Before experiments, chicken embryos were sacrificed and staged according to the number of somites present (Hamburger and Hamilton, 1992)[A]. Basilar papillae were isolated as described previously. The preparations were dissected in oxygenated chicken saline containing (in mM) 155 NaCl, 6 KCl, 4 $CaCl_2$, 2 $MgCl_2$, 5 HEPES, and 3 glucose at pH 7.4. The tegmentum vasculosum and the tectorial membrane were removed without any prior enzymatic treatment using a fine minutia needle. Chicken basilar papillae were stored in a 37°C incubator in Minimum Essential Medium (Invitrogen) before recordings from hair cell *in situ*. All experiments were performed at room temperature (21-23°C) within 5-45 min of isolation. All of the reagents were obtained from Sigma Chemicals, unless otherwise specified.

Electrophysiology. Ca^{2+} currents were recorded in whole-cell voltage-clamp configuration, using 3-5 ΩM resistance pipettes. Currents were amplified with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) and filtered at a frequency of 2-5 kHz through a low-pass Bessel filter. The data were digitized at 5-500 kHz using an analog-to-digital converter (Digidata 1200; Axon Instruments). The sampling frequency was determined by the protocols used. No online leak current subtraction was made, and as such only recordings with holding current less than 5 pA were accepted for analyses. The liquid junction potentials were measured and corrected. The capacitative transients were used to estimate the capacitance of the cell, as an indirect measure of the cell size. Membrane capacitance was calculated by dividing the area of the transient with

[A] Hamburger V, Hamilton HL (1992) A series of normal stages in the development of the chick embryo. Dev Dyn 195: 231-272.

the magnitude of the voltage step. Capacitative decay was fitted with a single exponential to determine the membrane time constant.

Series resistance was estimated from the membrane time constant, given its capacitance. This study includes ~367 cells with a series resistance (R_s) within the 5-20 MΩ range. After 60-90% compensation the mean residual, uncompensated R_s was 4.7±0.2 MΩ. The seal resistance was typically 5-20 GΩ. The liquid junction potentials were measured and corrected.

Action potentials were amplified (100X), filtered (bandpass 2-10 KHz), and digitized at 5-500 kHz using the Digidata 1200 (Axon Instruments) as described earlier. Extracellular solution for most experiments contained (in mM) NaCl 145, KCl 6, $MgCl_2$ 1, $CaCl_2$ 0-2, D-glucose 10, HEPES 10, pH 7.3. For perforated patch experiments, the tips of the pipettes were filled with the internal solution containing (in mM): KCl 150, HEPES 10, D-glucose 10, pH 7.3. The pipettes were front-filled with the internal solution and back-filled with the same solution containing 250 μg/ml amphotericin. The stock solutions of all toxins used were made either in ddH_2O or DMSO and stored at -20°C. The final concentration of DMSO in the recording bath solution was ~0.001%. Kurtoxin (Peptide Int.), mibefradil, and TTX were dissolved in ddH_2O. Stock solutions were reconstituted and perfused in the recording chamber.

To record Ca^{2+} currents, voltage-dependent outward K^+ currents were suppressed using a pipette solution containing NMG^+ and Cs^+ ions. Extracellular solution contained (in mM) NaCl 110, KCl 6, 4AP 5, $CaCl_2$ 5, TEA-Cl 25, D-glucose 10, HEPES 10, pH 7.3, 310 mOsm. Intracellular solution contained (in mM) NMG 70, CsCl 75, Na_2ATP 5, $MgCl_2$ 2, HEPES 10, EGTA 10, D-glucose 10, pH 7.3, 300 mosm. The stock solutions of all toxins/drugs used were made either in ddH_2O or DMSO and stored at -20°C.

Data analysis. The number of cells (*n*) is given with each data set. Data were analyzed using pClamp8 (Axon Instruments), Origin7.0 (Microcal Software) and Excel (Excel 2000, Microsoft). Time constants (τs) 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:

$I=I_0+A_1(\exp(-t/\tau_1)+A_2(\exp(-t/\tau_2)+A_n(\exp(-t/\tau n)$.

Where I_0 is the initial current magnitude, τ_1, $\tau_2 \ldots \tau_n$ are the time constants, and A1, A2…An, are the proportionality constants. Voltage-dependences of activation were examined from tail currents at different developmental stages (E10-P2), then normalized steady-state curves were fitted with the Boltzmann distribution**.** In addition, the steady-state inactivation curve was generated from normalized currents measured at a test potential following several conditioning pre-pulses on E10 hair cells. Pooled data were presented as mean ± SD. Significant differences between groups were tested using Student's *t*-test, with $P < 0.05$ or 0.01, indicating a statistically significant difference.

Reverse transcription PCR. Total RNA was extracted from micro-dissected basilar papilla of E8, E12, E16 and P1 chickens using RNA*later* (Ambion) and RNAeasy Mini Kit (Qiagen). Genomic DNA contamination was eliminated using RNase-free DNase treatment. For each age, 2µg of total RNA was reverse transcribed using oligo $(dT)_{24}$ primers (Ambion) and a SuperScript III reverse transcriptase (Invitrogen). RT-PCR was performed using *Taq* DNA Polymerase (Qiagen) and 1/20 of each cDNA preparation. Forty-five cycles (94°C for 45 s, 60°C for 45 s, and 72°C for 1 min) were conducted with primer pairs derived from chicken cDNA sequences as well as EST sequences, specifically, Ch1G-F2: 5'-CTCTTTGGCGACCTGGAGTGTG-3' and Ch1G-R2: 5'-TGCACCGATGCCTTTGAACC -3' (Ca_v3.1: 166-865 of BU413293.1), Ch1C-F1: 5'-CGCCTTGATTGTTGTGGGTAGC -3' and Ch1C-R1: 5'-GATCAGGAAGGCACAGAGCATG -3' (Ca_v1.2: 1-508 of AF027610), Ch1D-F1: 5'- GGCATAATGGCGTATGGTGG -3' and Ch1D-R1: 5'-TATGAAGGAATGCTCCGAAAGC -3' (Ca_v1.3: 2455-3154 of NM_205034), cGAPDH-F: 5'- CGTCCTCTCTGGCAAAGTCC -3' and cGAPDH-R: 5'-AGTTGGTGGTGCACGATGC -3' (glyceraldehyde-3-phosphate dehydrogenase,

GAPDH): 117-517 of NM_204305.1). All PCR products were then sequenced for identity confirmation.

Quantitative RT-PCR. Two-step quantitative real-time RT-PCR was performed using Brilliant SYBR Green QRT-PCR Reagents (Stratagene) by an ICycler IQ Real Time PCR Detection System (Bio-Rad). The thermal cycling is comprised of an initial step at 95°C for 10min, followed by 40 cycles of 30s at 95 °C, 30s at 60°C, and 1 min at 72°C. The primers used are Ch1G-qF: 5'- TCTTTGGCGACCTGGAGTG -3' and Ch1G-qR: 5'-CCATCTCCAGTTCTGCCTC-3' (Ca_v3.1: 167-477 of BU413293.1), Ch1C-qF: 5'-ACACGCCAGCATGATTTCC-3' and Ch1C-qR: 5'-GCGTGATGCGTCTTGTGAAG-3' (Ca_v1.2: 102-382 of AF027610), Ch1D-qF: 5'-CTGGCTACAGTGATGAAGAACC-3' and Ch1D-qR: 5'-AGCACAAGGTTGAAGAACAGG-3' (Ca_v1.3: 6742-7144 of NM_205034), cGAPDH-qF: 5'-CGTCCTCTCTGGCAAAGTCC-3' and cGAPDH-qR: 5'-AGTTGGTGGTGCACGATGC-3' (GAPDH: 117-517 of NM_204305.1), and Ch1G-LF-qF: 5'-GAAAGGCACTTGTTTGATAC-3' and Ch1G-LF-qR: 5'-CTGGTTCTCAGAGCTGTAAT-3' (specific for long transcript of Ca_v3.1). Serially diluted DNA fragments were prepared as standard solutions for absolute quantitative analysis of mRNA expression. Standard curves were drawn by plotting the threshold cycle (C_T) against serially diluted DNA concentrations in log copy number. The equation obtained from the standard curve shows the quantitative relationship between C_T and DNA concentration, which was used to calculate the number of transcript copy number per µg of total RNA. Melting curves were generated for each reaction to confirm the specificity of amplification and PCR efficiency was optimized to about 98-101% for each primer pair. For each age, three individual RNA preparations were analyzed and each reaction was done in triplet.

***In Situ* Hybridization** 114 bp new exon specific to the long isoform of Ca_v3.1 was amplified using primer Ch1G-LF-qF and Ch1G-LF-qR then subcloned into the pCRII-TOPO vector (Invitrogen). This fragment was labeled with digoxigen-UTP and the labeled probe was quantified following the manufacturer's manual (Roche Molecular BioChemicals). Temporal bones from E10, E16, and P3 chicks were fixed in 4% PFA.

The cochleae were removed and placed into PFA/sucrose O/N. Cryosections (10 μm) were hybridized at 60°C (probe concentration 1ng/μl) then washed in 50% formamide/2 x SSC (62°C) with final stringency washes in 0.2 x SSC (37°C). Detection was performed using anti-DIG antibodies (1:500, Roche) and visualized using NBT/BCIP. Images were captured using a Leica DMLB microscope equipped with a Spot RT color CCD camera.

Fig. 6. Pharmacology of transient Ca^{2+} currents in developing hair cells. (*A-D*) We examined the sensitivity of the transient Ca^{2+} current to 100 nM kurtoxin (*A*) (also see Fig. 2), 10 μM nickel (*B*) and 100 nM mibefradil (*C*) at E10 (*Top*), E12 (*Middle*) and E16 (*Lower*). Control current traces are shown in faint solid lines; the current remaining after application of kurtoxin, nickel and mibefradil are shown in dashed lines; and the difference currents, which reflect drug-sensitive components, are represented in dark solid lines. (*D*) Histogram showing that the Ca^{2+} current component that was sensitive to membrane holding potential (I_{hp}) at E10, E12 and E16 was not significantly different from the kurtoxin-, nickel- and mibefradil-sensitive current [$P > 0.05$; n = 8 (kurtoxin); n = 7 (nickel); n = 7 (mibefradil)].

Fig. 7. Time constants of activation and deactivation do not differ over different stages of development. (*A-C*) Activation time constant of Ca^{2+} currents was similar at all stages in development, on the order of submiliseconds activation time constant (for -90 mV test potential, E12, n = 22; E16, n = 21; P3 n = 11. (*D-F*) There were two different time constants of deactivation (submilisecond and ~8 ms) observed across all stages of development, implicating the possible presence of multiple currents.

Fig. 8. Kinetics of inactivation of Ca^{2+} currents in the developing hair cell (*A*) Normalized current traces elicited using ~70-s pulse for E12 (solid black line) and P2 (gray line) cells. The fast components of decay were absent at P2. (*B*) Current decay could be best fit with three different time constants (τ) at E12 stage of development, and a single τ (in milliseconds) at mature stages [τs for E12: 51 $\pm$ 17, 325 $\pm$ 132, 7,040 $\pm$ 2,564 (*n* = 11 cells); and τ for P2; 8,750 $\pm$ 3,447; (*n* = 4 cells)]. For the example shown at E12, the exponential fits yielded $\tau_1 = 37$, $\tau_2 = 345$, and $\tau_3 = 7{,}144$. The current trace illustrated from the P2 HC was fitted with τ = 8,692 ms. (*C*) Components of the Ca^{2+} currents were sensitive to holding voltages. The normal resting potentials of developing HCs range between ~-50 to -65 mV, which fell within the voltage ranges at which a substantial portion of the inactivating current component was not available for activation. We examined the time-dependence of development of inactivation after varying durations at -50 and -90 mV. The time course of development of inactivation at -90 mV (*Left*) and

the τs of development of inactivation were determined (*Right*) for current traces recorded at E12 and P2 [τs for E12: 27 ± 5, 104 ± 13, 1,164 ± 244 ms (*n* = 9 cells); τ for P2: 298 ± 75 ms (*n* = 7 cells)]. At E12, ~55% of the current was available for activation after 5-sec at -50 mV, and the kinetics of development of inactivation required three time constants to reflect accurately the time course of current inactivation. By E16, ~65% of the current was available for activation, and two time constants were sufficient to fit the time course of the development of inactivation. (*D*) Examples of traces generated to examine the time course of the development of inactivation at -50 mV of E12 currents (*Left*). The right shows the time course and the exponential fits [in solid lines, τs for E12: 48 ± 17, 874 ± 321, 4,412 ± 905 ms (*n* = 5 cells); for P2: the current was sustained]. The inter-pulse interval for these recordings was ~80 s. Amplitude of the currents were normalized to the amplitude of the currents activated from a holding potential of -90 mV and plotted against the duration of depolarizing step to -50 mV. By P2, virtually 100% of the current was available for activation. To estimate how quickly the current recovered from inactivation, we measured recovery kinetics after 3-seconds at the holding potential of -50 mV, using standard recovery time protocol. Traces of currents used to determine the rate of recovery from ~5 seconds at -50 mV. We measured the amplitude of Ca^{2+} currents by depolarizing to a fixed potential, after variable time at -90 mV. Amplitude of these currents was normalized to the amplitude of the currents activated from -90 mV and plotted against the duration of steps to -90 mV. (*E*) (*Left*) An example of such protocol on an E12 cell is shown. (Right) The exponential fits to the data were; τs for E12 at -90 mV: 40 ± 14, 1,185 ± 252 ms (*n* = 7); for E16 at -90 mV: 1,787 ± 343 ms (*n* = 6 cells).

Fig. 9. Effects of T-type Ca^{2+} channel blockers on SAPs during HC development and regeneration. The sensitivity of SAPs in developing HCs to kurtoxin, nickel and mibefradil was tested at E12 and E16 and in regenerating HCs after gentamicin posttreatment day 7 (PT7). (*A* and *B*) The T-type Ca^{2+} current blockers kurtoxin (40 nM) reduced the spike frequency of SAP in HCs at E12 (*A*) and E16 (*B*) For the examples shown, the mean spike frequency (in Hz) for control at E12 and E16 were 5.8 and 4.0, respectively. After application of 40 nM kurtoxin, the mean spike frequencies at E12 and

E16 were reduced to 2.3 and 2.4, respectively. Summary data of spike frequencies for control SAPs at E12 and E16 were 4.5 $\pm$ 1.5 Hz (n = 4 cells) and 6.8 $\pm$ 3.1 (n = 3 cells), respectively. After application of 40 nM kurtoxin the spike frequencies were at E12 2.1 $\pm$ 0.7 Hz (n = 4 cells) and at E16 2.9 $\pm$ 1.1 Hz (n = 3 cells). (*C*) Application of 100 μM Ni^{2+} completely blocked SAP in two cells tested at E12. (*D*) Mibefradil also reduced spike frequency of SAPs that ensue in regenerating HCs. Mean of control spike frequencies at PT7 were 2.1 $\pm$ 0.6 (n = 5 cells) and after application of 100 nM mibefradil the spike frequencies dropped to 0.6 $\pm$ 0.3 (n = 5 cells). The dotted lines denote 0 mV.

Fig. 10. Spontaneous action potentials were dependent on external Ca^{2+}. (*A*) Spike activity in E12 hair cell was abolished in the absence of external Ca^{2+}. Indeed spike amplitudes increased as the external Ca^{2+} was changed from 2 to 5 mM. (*B*) The effects of increased external Ca^{2+} on spike frequency, amplitude, and the rate of change of depolarization and repolarization. Further, the Ca^{2+} dependence of SAPs could be seen when whole-cell patches, containing 500 μM EGTA and perforated patches were used (*C*). Data from five cells at E10 are as follow: mean spike frequency within burst (Hz), 500 μM EGTA, 2.3 $\pm$ 1.9; perforated patch, 1.4 $\pm$ 0.8: and mean inter-spike interval across the duration of recording (s), 500 μM EGTA, 0.7 $\pm$ 0.5; perforated patch, 3.1 $\pm$ 1.7; n = 7).

Fig. 11. Distinct firing pattern of developing and regenerating hair cells. Current clamp recordings illustrate three different examples of hair cells based on their burst discharge. (*A*) HCs with tonic action potential discharge with inter-spike intervals (*Left*) described by a symmetrical binomial distribution (*Right)* were classified as non-burst cells. (*B*) Typical burst HCs produce a high frequency, transient action potential discharge in clusters (*Left*) leading to a strongly skewed distribution (R*ight*). (*C*) (*Left*) Atypical burst was used to describe HCs with action-potential discharge pattern that was initially a modest frequency, then had a high frequency discharge, followed by regular inter-spike intervals. (*Right*) Characteristic distribution of an atypical burst HC.

Fig. 12. Discharge pattern of action potentials in hair cells was altered during development. (*A-D***)** Examples of changes in bursting activity in developing hair cells from E8 to E16 are shown E8 (*A*), E10 (*B*), E12 (*C*), E16 (*D*). *E-G*. Summary data of the duration of burst (*E*), inter-burst intervals (*F*), and the spike frequency within a burst (*G*) at different stages in development (E8-E16) are illustrated in the histograms.

Fig. 13. RT-PCR analysis of three different VGCC channels, $Ca_v3.1$, $Ca_v1.2$, and $Ca_v1.3$, were performed with total RNA from basilar papilla of E8, E12, E16 and P1 chicken, respectively. The same analysis of GAPDH was performed in parallel as control for production of cDNA. While two transcripts were detected for $Ca_v3.1$, only one was detected for $Ca_v1.2$ and $Ca_v1.3$. Differential expression during development was observed for each transcript. The experiments were repeated twice and similar findings were observed. NTC: no cDNA template control.