Developmental changes in two voltage-dependent sodium currents in utricular hair cells

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Abstract

Two kinds of sodium current (I_{Na}) have been separately reported in hair cells of the immature rodent utricle, a vestibular organ. We show that rat utricular hair cells express one or the other current, depending on age (between postnatal days 0 and 22, P0-P22), hair cell type (I, II, or immature), and epithelial zone (striola vs. extrastriola). The properties of these two currents, or a mix, can account for descriptions of I_{Na} in hair cells from other reports. The patterns of Na channel expression during development suggest a role in establishing the distinct synapses of vestibular hair cells of different type and epithelial zone.

All type I hair cells expressed I_{Na,1}, a TTX-insensitive current with a very negative voltage range of inactivation (midpoint −94 mV). I_{Na,2} was TTX-sensitive and had less negative voltage ranges of activation and inactivation (inactivation midpoint −72 mV). I_{Na,1} dominated in the striola at all ages, but current density fell by two-thirds after the first postnatal week. I_{Na,2} was expressed by 60% of hair cells in the extrastriola in the first week, then disappeared. In the third week, all type I cells and about half of type II cells had I_{Na,1}; the remaining cells lacked sodium current. I_{Na,1} is probably carried by Na_{V}1.5 subunits, based on biophysical and pharmacological properties, mRNA expression, and immunoreactivity. Na_{V}1.5 was also localized to afferent somata and calyx endings on type I hair cells. Several TTX-sensitive subunits are candidates for I_{Na,2}. 


Introduction

Hair cell receptor potentials are modulated by diverse voltage-gated ion channels in the basolateral membrane. Much attention has been paid to the potassium (K+) channels, which dominate numerically, and calcium (Ca^{2+}) channels, which participate in synaptic transmission. Information on voltage-gated sodium (Na+) channels is more fragmentary. By the classic properties of voltage range of inactivation and sensitivity to tetrodotoxin (TTX), voltage-gated Na^+ currents in hair cells fall into three classes: TTX-sensitive currents that inactivate at very negative potentials (Evans and Fuchs 1987; Sugihara and Furukawa 1989; Witt et al. 1994; Masetto et al. 2003); TTX-sensitive currents that inactivate at less negative potentials (Chabbert et al. 2003; Marcotti et al. 2003); and TTX-insensitive currents that inactivate at very negative potentials (Oliver et al. 1997; Rüsch and Eatock 1997; Géléoc et al. 2004).

Two kinds of Na^+ current have been described in immature rodent vestibular and cochlear hair cells, but never in one report. A TTX-insensitive, very negatively inactivating current was reported by Rüsch and Eatock (1997) in hair cells of the immature mouse utricular macula (P0-P10). In the same preparation, Géléoc et al. (2004) observed that this current peaked at embryonic day (E) 16 and decreased dramatically by P0 (E20). A Na^+ current in outer hair cells of the immature rat cochlea has similar TTX sensitivity and voltage dependence (P0-P11, Oliver et al. 1997). More recently, however, related preparations – the immature rat utricular macula and inner hair cells of the immature mouse cochlea – have yielded a TTX-sensitive and less-negatively inactivating Na^+ current (Chabbert et al. 2003; Marcotti et al. 2003). Did experimental manipulations alter the currents’ properties? Does expression differ between rats and mice? Or was Na^+ current heterogeneity missed? For the utricle, our results favor the last explanation. We show that in the rat utricular macula, both currents are expressed between P0 and P9, but in different hair cells; which current is expressed varies with postnatal age, location in the epithelium, and hair cell type.
Voltage-gated Na\(^+\) channels comprise pore-forming (\(\alpha\)) subunits and sometimes accessory (\(\beta\)) subunits that can modulate the behavior or expression of the \(\alpha\) subunits. Chabbert et al. (2003) provided RT-PCR evidence for multiple TTX-sensitive \(\alpha\) subunits in individual immature rat utricular hair cells. Here we show that the TTX-insensitive current is likely to be carried by Na\(_{\alpha1.5}\) subunits, originally described in heart muscle (Rogart et al. 1989).

In cochlear hair cells, a developmental decline in Na\(^+\) current amplitude coincides with changes in Ca\(^{2+}\) and K\(^+\) channels, with the net effect being a reduced tendency to fire action potentials (Marcotti et al. 2003). These changes occur around the onset of hearing and may mark the transition from a non-sensing epithelium, in which spikes contribute to development, to a sensing epithelium, in which spiking may interfere with outer hair cell electromotility or the graded representation of sounds. In the rat utricular macula, we see a comparable reduction in Na\(^+\) currents with a similar time frame, reflecting complete loss of the TTX-sensitive current and a decline in size of the TTX-insensitive current.

**Materials and Methods**

Procedures involving animals were approved by the Institutional Animal Care and Use Committees at Baylor College of Medicine and the University of Illinois at Chicago. Compounds were obtained from Sigma (St. Louis, MO) unless otherwise specified.

**Electrophysiology**

*Preparations.* Most recordings were from hair cells in excised, semi-intact preparations of the rat utricular epithelium, prepared as described previously for the mouse utricular macula (Vollrath and Eatock 2003). The sensory part of the epithelium is called the macula. Some recordings were obtained from solitary hair cells isolated from the rat utricular macula, as described previously (Wong et al. 2004).
In both types of experiment, Long-Evans rats (P0-P22, Charles River Laboratories, Wilmington, MA) were anesthetized by cooling (<P4) or intraperitoneal injection of pentobarbital sodium (Nembutal, 50 mg/kg) and decapitated. The remainder of the dissection was carried out in our standard external solution (K⁺-SES, see *Solutions*). The head was bisected, the brain was removed and the otic capsule was opened to expose the membranous inner ear. The endolymphatic compartment of the utricle was opened and bathed for 10 min at room temperature in extracellular solution containing 100 µg/ml protease XXIV, which facilitated removal of the otolithic gel overlying the hair bundles. The utricular epithelium was excised from the otic capsule into a chamber containing K⁺-SES.

For the semi-intact preparation, the epithelium was trimmed, affixed with CellTak (BD Biosciences, Bedford, MA) to a coverslip in a glass-bottomed experimental chamber, and observed at ×630 or ×1000 on a fixed-stage upright microscope (Axioskop FS, Carl Zeiss, Thornwood NY) with water-immersion objectives and differential interference contrast optics.

For a minority of experiments we used isolated hair cells. To obtain these, we placed the excised utricular epithelium in K⁺-SES containing crude papain (500 µg/ml) and L-cysteine (300 µg/ml) for 40-60 min at 37°C. The epithelium was then transferred to external solution containing bovine serum albumin (500 µg/ml) for 10 minutes at room temperature (22-24°C), and finally to a recording chamber fit with a glass coverslip. The hair cells were mechanically dispersed with a fine probe and viewed at ×400 or ×600 on an inverted microscope with differential interference contrast optics (IMT-2, Olympus Corporation, Lake Success, NY).

*Solutions.* The potassium standard external solution (K⁺-SES) used for dissections contained (mM): NaCl (144), NaH₂PO₄ (0.7), KCl (5.8), CaCl₂ (1.3), MgCl₂ (0.9), D-glucose (5.6), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES; 10), vitamins and amino acids as in Eagle's MEM (Minimal Essential Medium); pH 7.4 with ~7.5 mmol NaOH, ~310 mmol/kg.
For voltage clamp recording of Na\(^+\) currents, the standard external solution was altered to minimize K\(^+\) currents by adding the K\(^+\) channel blocker, 4-aminopyridine (4-AP) and replacing K\(^+\) with the less permeant ion, Cs\(^+\). The standard external solution for recording (Cs\(^+\)-SES) contained (mM): NaCl (130), 4-AP (5), CsCl (5), MgCl\(_2\) (1), CaCl\(_2\) (5), HEPES (10), D-glucose (5.6), vitamins and amino acids as in Eagle’s MEM, pH 7.4 with ~2.5 mmol NaOH, ~310 mmol/kg. The experimental chamber was constantly perfused with Cs\(^+\)-SES. In several voltage clamp experiments, recordings were made in K\(^+\)-SES with 1.3 mM CaCl\(_2\) and 0.9 mM MgCl\(_2\); Na\(^+\) currents were isolated kinetically from the slower K\(^+\) currents.

We used the ruptured-patch method of whole-cell recording. In voltage-clamp experiments of Na\(^+\) currents, the electrode usually contained the standard internal solution (Cs\(^+\)-SIS) comprising (mM): CsCl (135), MgCl\(_2\) (3.5), HEPES (5), EGTA (0.2), Na\(_2\)ATP (5), Li\(_x\)GTP (0.1), Na-cAMP (0.1), pH 7.4 with ~5 mmol CsOH, ~280 mmol/kg. For current clamp recordings, the external solution was K\(^+\)-SES and the electrode contained K\(^+\)-SIS (mM): KCl (140), MgCl\(_2\) (3.5), Na\(_2\)ATP (2.5), HEPES (5), EGTA (10), Na-cAMP (0.1), Li\(_x\)GTP (0.1), pH 7.4 with KOH (~25 mM), ~290 mmol/kg.

**Recordings.** All recordings were performed at room temperature (22-25°C). For the semi-intact preparation, we pulled recording electrodes from R-6 glass (Garner Glass Co., Claremont, CA); electrodes had resistances ranging from 2.5 to 4 M\(\Omega\) in our standard solutions. We used the same method to record from hair cells as described in Vollrath and Eatock (2003) for hair cells in the semi-intact mouse utricular macula: We lowered the recording pipette into the epithelium ~30 \(\mu\)m from the cell of interest, maintaining positive pressure on the pipette in order to clear a path for the electrode’s advance within the epithelium. The electrode was advanced in between supporting and hair cells to its target membrane; if the hair cell of interest had a calyx, that calyx was dying because contact had been severed with the ganglion cell bodies and it was relatively easy to dislodge the calyx from the hair cell membrane. Acquisition of a hair cell was confirmed
visually by focusing up and down from hair bundle to pipette location; also, at the end of each recording, the pipette lifted its attached hair cell - with visible bundle - out of the epithelium. Ionic currents were recorded in whole-cell mode with the EPC-10 amplifier with an integrated interface (HEKA Instruments Inc., Southboro, MA), controlled by a Pentium IV PC (Dell Inc., Round Rock, TX) running Pulse 8.62 software (HEKA). Currents were filtered with an integrated 4-pole Bessel filter at 8.3 kHz. Average residual series resistance, after electronic compensation, was \(4.2 \pm 0.14\) M\(\Omega\) (range 1.3 - 13.4 M\(\Omega\), \(n = 184\) cells). The mean clamp rise time (the product of residual series resistance and cell capacitance) was \(27 \pm 0.9\) \(\mu\)s (range 9.4 - 84.2 \(\mu\)s, \(n = 184\)). Potentials are corrected for liquid junction potentials, calculated with JPCalc software (Barry 1994), of \(-7.4\) mV between Cs\(^+\)-SES and Cs\(^+\)-SIS and \(-5.4\) mV between K\(^+\)-SES and K\(^+\)-SIS. Thus, the holding potential in Cs\(^+\)-based solutions was \(-67.4\) mV. Corrections for liquid junction potentials were also applied to voltages recorded in current clamp mode. All recorded currents were leak-subtracted with a \(+P, -P/4\) protocol (Armstrong and Bezanilla 1974); the original, non-subtracted data were also stored. Data were analyzed with PulseFit 8.62 (HEKA), Origin 7.0 (OriginLab Corp., Northampton, MA) and Excel 2002 (Microsoft, Redmond, WA) software.

Hair cells in the semi-intact preparation were classified according to their macular location and cell type. The utricular macula in mammals has a quasi-central stripe, the striola, with distinctive morphology and physiology (illustrated in Fig. 6; reviewed in Eatock and Lysakowski 2006). Hair bundles reverse orientation within the striola. Location was defined relative to the line of bundle reversal: hair cells within three cells of the reversal line were considered striolar and cells further than six cells from the line of reversal were considered extrastriolar, based on calretinin staining of calyx-only afferents (Desai et al. 2005b). Hair cells at intermediate locations and at the far edges of the epithelium were avoided. The parts of the extrastriola lateral and medial to the striola were distinguished as illustrated in the inset to Fig. 6.
In amniotes, mature vestibular hair cells are classified as type I or type II. The two types differ in multiple ways, notably in the expression of K⁺ channels and in the form taken by afferent terminals (reviewed in Eatock and Lysakowski 2006). Type I hair cells have an unusually large and negatively activating K⁺ conductance, g_K,L, and are contacted by large calyceal terminals. Type II cells have smaller K⁺ conductances at resting potential and are contacted by bouton terminals. In most of our electrophysiological experiments, we could not use K⁺ currents to distinguish hair cell type, as they were blocked. Instead, we classified hair cells as type I if they were contacted by calyces and as type II if they were from P9 or older animals and lacked calyces. In 184 cells, there were 50 type I (39 striolar, 11 extrastriolar) and 29 type II, leaving 105 cells unclassified. Although a few calyces are seen in the rat utricular macula as early as P0 (Gaboyard et al. 2003), in our electrophysiological sample, most (43/50) hair cells with calyces came from animals aged P3 or older. The seven cells with calyces between P0 and P2 all came from the striola, consistent with its faster development (Sans and Chat 1982; Rüsch et al. 1998).

For recording from isolated hair cells, we pulled recording electrodes from filamented thin-walled glass (TW150F-4, WPI, Inc., Sarasota, FL); pipette resistances were 1-3 MΩ in standard recording solutions. Recordings were made with the ruptured-patch method and similar solutions to those used in the semi-intact preparation. Currents were amplified with an Axopatch 200B amplifier and digitized by a DigiData 1200 interface (Axon Instruments Inc., Foster City, CA) controlled by Clampex 8 software (Axon). The amplifier output was low-pass filtered at 10 kHz. Data were analyzed with Clampfit 8 (Axon), Origin 7.0 (OriginLab) and Excel 2002 (Microsoft) software. Isolated hair cells were identified as type I or II by cell shape.

Analysis. Results are expressed as means ± S.E.M. Significance was determined with the Students’ t-test or one-way or two-way ANOVAs with post-hoc Tukey means comparisons, as implemented by Origin software.

To generate activation curves (conductance-voltage, g(V), relations) for Na⁺ current, we converted peak inward currents to conductances by dividing by the driving force, (V−E_Na), where
$V$ is membrane potential, and $E_{Na}$ is the Na\(^+\) equilibrium potential (+65 mV for the Cs\(^+\)-based solutions and +80 mV for the K\(^+\)-based solutions). Calculated conductances were plotted against voltage and the resulting curves were fit with a single Boltzmann function (Eq. 1):

$$g(V) = \frac{g_{\text{max}}}{1 + e^{(V_{1/2} - V)/S}}$$  \hspace{1cm} (1)

where $g_{\text{max}}$ is the maximum conductance, $V_{1/2}$ is the voltage corresponding to half-maximal activation (the midpoint of the Boltzmann function), and $S$ is the voltage range over which conductance increases $e$-fold before it begins to saturate.

Na\(^+\) currents are rapidly inactivating. To study the voltage dependence of inactivation, we stepped to a near maximally activating voltage after an iterated prepulse potential and plotted the peak current as a function of prepulse voltage (*inactivation curves*). The curves were fit by a single Boltzmann function:

$$I(V) = \frac{I_{\text{max}}}{1 + e^{(V - V_{1/2})/S}} + I_o$$  \hspace{1cm} (2)

where $I_o$ is the offset current and $I_{\text{max}}$ is the maximum current; or a double Boltzmann function:

$$I(V) = \frac{I_1}{1 + e^{(V - V_1)/S_1}} + \frac{I_2}{1 + e^{(V - V_2)/S_2}} + I_o$$  \hspace{1cm} (3)

where $I_1$ and $I_2$ are the maximum currents, $V_1$ and $V_2$ are the $V_{1/2}$ values, $S_1$ and $S_2$ are the slope values for each term.
The midpoints ($V_{1/2}$ values) of single Boltzmann fits to inactivation curves had a bimodal distribution; each component was fit with a Gaussian function (Eq. 4):

$$N = \frac{A}{w/\sqrt{\pi}/2} e^{-\frac{(V_{1/2} - V_C)^2}{w^2}}$$

(4)

where $N$ is the number of cells, $V_C$ is the $V_{1/2}$ value at the center of the function; $w$ is the width of the curve (mean ± 1 standard deviation), and $A$ is the total number of cells under the curve.

The inactivation time course was fit with a single exponential function (Eq. 5):

$$I(t) = I_0 + Ae^{-t/\tau}$$

(5)

where $I$ is current, $t$ is time, $I_0$ is the steady state current, $A$ is the amplitude and $\tau$ is the time constant. The recovery from inactivation was fit either with Eq. 5 or with a double exponential function:

$$I(t) = I_0 + A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2}$$

(6)

where $A_1$ and $A_2$ are amplitudes and $\tau_1$ and $\tau_2$ are time constants for each term.

**RT-PCR**

We used the reverse-transcription polymerase chain reaction, RT-PCR, method to test for the expression of mRNA corresponding to nine known Na$^+$ channel $\alpha$ subunits and four $\beta$ subunits (Table 1). We studied utricular maculae and the sensory epithelia (cristae) of all three
semicircular canals from P1 and P21 rats. In some cases, vestibular ganglia were used as a positive control (see below).

The maculae and cristae were prepared as described for the semi-intact preparation for electrophysiology, with the following additional steps. After the protease treatment and removal of the otolithic gel from the utricle, we excised the utricle with attached anterior and horizontal ampullae (and sometimes the posterior ampulla) into a dish of standard external solution containing 500 \(\mu\)g/ml thermolysin (Protease X; 37°C for 1 h), which facilitates the separation of the epithelia from the basement membranes (Saffer et al. 1996). The superior division of the vestibular ganglion was also excised. The epithelia were peeled from the basement membranes by lifting with a fine eyelash and the peeled epithelia and ganglia placed in separate RNase/DNase-free tubes. Excess liquid was removed and the tissue samples were placed on dry ice.

We used the RNeasy kit (QIAGEN, Valencia, CA) to isolate RNA from utricular maculae (three pooled at a time), cristae (six pooled at a time) and vestibular ganglia (one at a time). The tissue was homogenized in lysis buffer with a mortar and pestle and a QIAshredder column (QIAGEN). An equal volume of 100% ethanol was added, the combined solution was placed on an RNeasy column, washed with RW1 solution (QIAGEN), and the RNA was eluted in RNase-free H\(_2\)O. The resulting RNA solution was reverse-transcribed to complementary (c) DNA using the Advantage RT-for-PCR Kit (BD Biosystems, Palo Alto, CA) with the Moloney murine leukemia virus (MMLV) reverse transcriptase and random hexamer primers.

We controlled for possible contamination of samples by genomic DNA in several ways:

1) Primer design: Primer sets were designed across intron-exon boundaries as determined from rat sequences. Genomic DNA contamination would be revealed as higher molecular weight bands than the predicted PCR product. None were observed.

2) Tissue treatment: During the RNA isolation process, tissue homogenization helped break up genomic DNA. In addition, RNeasy columns contain a silica membrane that is designed to eliminate most DNA from the sample. Residual DNA was removed by
treating the column with RNase-free DNase I (QIAGEN) for 30 min before eluting the RNA.

3) Negative RT controls: For each primer set, samples of inner ear tissue were prepared as described above, substituting water for the MMLV reverse transcriptase. Thus, only genomic DNA would be present as a template for amplification. No bands were observed on agarose gels (data not shown).

PCR was done with a PTC-100 thermocycler (MJ Research, Reno, NV) using the TAQ enzyme (Applied Biosystems, Foster City, CA) and the various primer sets (IDT, Coralville, IA) listed in Table 1. A “hot start” (94°C for 4 min) reduced mis-priming. The PCR protocols comprised 40 cycles of 94°C for 1 min, 58-62°C (annealing temperature) for 30 s, and 72°C for 35 s; plus a final 7 min at 72°C. PCR products were resolved on 1.2% agarose gels and visualized with ethidium bromide.

The PCR product for each primer set was sequenced at least once; products were sequenced from the utricular macula where present, or otherwise from vestibular ganglia (SeqWright, Houston, TX). Each primer set was tested on each tissue type (utricular macula, crista) 2-14 times. For all but three primer sets, we simultaneously tested for expression in standard tissues (brain, heart or skeletal muscle), as a control for primer quality. NaV1.7, 1.8 and 1.9 are weakly expressed if at all in these standard tissues. In contrast, we found robust expression in vestibular ganglia, which we confirmed by direct sequencing. We therefore used the vestibular ganglia as our ‘control’ tissue for the NaV1.7, 1.8 and 1.9 primer sets. As a positive control for sample tissue quality, we tested for expression of the L-type Ca^{2+} channel α subunit, CaV1.3 (α1D), which we have found to be robustly expressed in maculae, cristae and, contrary to our initial observations (Bao et al. 2003), in vestibular ganglia. The same CaV1.3 primers were used as in Bao et al. (2003; Table 1).

**Single-cell RT-PCR.** We collected individual hair cells for RT-PCR analysis from intact P1 epithelia prepared as for electrophysiological experiments. Electrode glass was cleaned and
treated with RNaseZAP, rinsed with nuclease-free water and dried overnight. Low resistance pipettes (~1 MΩ) were pulled and the tips filled with ~8 µl K⁺-SIS. Individual cells of known region and cell type were sucked into the pipette; we did not record from them. Each cell was immediately placed in 1 µl RNase inhibitor and flash-frozen at −80°C.

Reverse transcription of RNA and amplification of specific DNA products were achieved using One-step RT-PCR (QIAGEN). To amplify PCR products corresponding to Naᵥ1.2 or Naᵥ1.5 subunits, we used a degenerate primer set (based on Chabbert et al. 2003) for the first round of amplification and subunit-specific primers for the second round (Table 1). To check the viability of individual cells, we included a primer set for β-actin, as well as the Na⁺ channel degenerate primer set, in the first round PCR. The PCR protocols were: (1) first round: 55°C for 30 min (for reverse transcription); 95°C for 15 min; 40 cycles of 94°C for 1 min, 52°C for 1 min, 72°C for 1 min; 72°C for 10 min; (2) second round: 95°C for 15 min; 40 cycles of 94°C for 1 min, 52°C for 1 min, 72°C for 1 min; 72°C for 10 min. Each PCR product was sequenced as described for whole epithelia.

**Immunohistochemistry**

Long-Evans rats of various ages (P0-P21) were deeply anesthetized with Nembutal (80 mg/kg), then perfused transcardially with 10-100 ml physiological saline containing heparin (2000 IU), followed by 2 ml/g body weight of an aldehyde fixative (4% paraformaldehyde, 1% acrolein in 0.1 M Phosphate Buffer (PB) with 1% picric acid and 5% sucrose, pH 7.4). Vestibular epithelia were dissected out in PB and cryo-protected in 30% sucrose-PB. Otoconia were eliminated with undiluted Cal-Ex for 1–10 min (Fisher Scientific, Pittsburgh, PA). Background fluorescence was reduced by incubating the tissues in 1% aqueous solution of sodium borohydride for 10 min. Frozen sections (35 µm) were cut with a sliding microtome.
Antibodies were from Chemicon (Temecula, CA) unless otherwise specified. Immunocytochemistry was done on free-floating sections or whole organs, permeabilized with Triton X-100 in a blocking solution of 0.5% fish gelatin and 1% BSA in phosphate-buffered saline (PBS). Samples of vestibular tissues were incubated with Triton X-100 at conditions that varied with postnatal age: P0-P1: 0.3% overnight at 4°C; P3-4: 0.5% for 1 h at room temperature (RT); P6-P8: 2% for 1 h at RT; P21: 4% for 1 h at RT. (For the younger ages, morphology was better preserved by decreasing the detergent concentration and increasing the incubation time.) Samples were then incubated with a cocktail of two primary antibodies diluted in the blocking solution: goat anti-calretinin and rabbit antibody against NaV1.5 (1:200) or NaV1.2 (1:75; Sigma, St. Louis, MO) or NaV1.6 (1:200) for 2 days at 4°C with 0.1%, 0.3% and 0.5% Triton X-100 for P0-P1, P4-P21 sections, respectively. We used calretinin antibody as a marker of immature hair cells (P0-P6), type II cells (P8-P21) and calyx afferents (P4-P21). (The calretinin label is not always shown.) Specific labeling was revealed with a cocktail of two secondary antibodies: fluorescein-conjugated donkey anti-goat and rhodamine-conjugated donkey anti-rabbit (1:200 in the blocking solution). Sections were rinsed with PBS between and after incubations and mounted on slides in Mowiol (Calbiochem, Darmstadt, Germany). The sections were examined at an optical section thickness of 0.4 – 1 µm, depending on the magnification, on a laser scanning confocal microscope (LSM 510 META, Carl Zeiss, Oberkochen, Germany). Final image processing was done with Adobe Photoshop software (San Jose, CA).

For each antibody, we did control reactions to test for non-specific labeling with no primary antibody and with primary antibodies pre-incubated with their antigenic peptide (10 µg/1 µg antibody) for 2 h at room temperature. Images comparing staining in control and test conditions were acquired and digitally processed identically. We also did Western blots on inner ear epithelia obtained from five adult rats (> P30; 250-300 g; NaV1.5) or six P10 rats (NaV1.2 and NaV1.6) to check that the antibody recognized a protein of the appropriate size. Membrane proteins were isolated from control tissues (heart, cerebellum, liver) using a method adapted from
Moore et al. (1998). Inner ear tissues were homogenized separately in the same homogenization buffer used for the membrane preparations. Aliquots containing 50 µg of protein homogenate were mixed with 5× SDS loading buffer. The inner ear tissue was incubated at 37°C for 5 min, while the control tissues were boiled for 5 min, then both were loaded onto 4-15% Tris-HCl mini-gel wells. After electrophoresis, the proteins were transferred from the gel onto nitrocellulose membrane overnight. The membrane was then blocked with 5% milk solution for 2 h, incubated in primary antibody solution for 2 h (1:200), incubated in secondary antibody solution for 1 h (1:30,000), and washed thoroughly with TBS-Tween. Bands were visualized with chemiluminescent detection (Amersham, Little Chalfont, UK). Western blots for all three Na⁺ channel antibodies (data not shown) had bands at the appropriate size in the inner ear and positive control tissues (heart and cerebellum) and not in the negative control tissue (liver): Na₉1.2, 228 kDa; Na₉1.5, 227 kDa; Na₉1.6, 226 kDa. For the Na₉1.2 and Na₉1.5 antibodies, we further showed that the band at the correct size was selectively blocked by pre-absorption with the antigenic peptide.

Results

Two voltage-gated Na⁺ currents in immature rat utricular hair cells

Transient current in hair cells required external Na⁺ Whole-cell currents were recorded in situ from 184 hair cells in the utricular maculae of rats between P0 and P22. The external and internal solutions were designed to minimize K⁺ currents. In almost all cells (91%, 167/184), depolarizing pulses following a hyperpolarizing step evoked rapidly activating and inactivating inward currents (Figs. 1A, 2A), often accompanied by a small steady current (Fig. 1A). The peak inward current is plotted as a function of voltage in Figure 1E. Replacement of external Na⁺ with the impermeant cation, N-methyl-D-glucamine⁺, NMDG⁺, eliminated the transient component
(n = 4 cells, P1, Fig. 1B), showing that it was carried by Na⁺. As shown later, the transient current was also sensitive to the Na⁺ channel blocker, TTX.

The residual inward current in NMDG⁺ (Figs. 1B,E) was carried by voltage-gated Ca²⁺ channels that show little rapid inactivation (Bao et al. 2003). It was difficult to eliminate Ca²⁺ current in these cells for two reasons. First, one component of the Na⁺ current was sensitive to the standard Ca²⁺ channel blocker, cadmium (Cd²⁺, Wooltorton et al. in press). Second, the Ca²⁺ current in mammalian vestibular hair cells is only partly blocked by high doses of dihydropyridines (Bao et al. 2003; Dou et al. 2004). Thus, most of our records include a small Ca²⁺ current component; as discussed later (Effect of Ca²⁺ current), it had little impact on our Na⁺ current measurements.

The NMDG⁺-sensitive current (Fig. 1D) included, in addition to the expected transient inward current, a small sustained component which may be a ‘persistent’ Na⁺ current similar to that described in mammalian neurons (e.g., Crill 1996; Do and Bean 2004; Vreugdenhil et al. 2004) and expression systems (e.g., Qu et al. 2001; Mantegazza et al. 2005). In four isolated rat utricular hair cells, the maximum persistent current was 10 ± 2.8% of the peak transient NMDG⁺-sensitive (difference) current. This is a larger percentage than reported for persistent currents in other cell types (1.5 - 5%). In these hair cells, some of the steady-state NMDG⁺ difference current may have been Ca²⁺ current that ran down during the NMDG⁺ application and so appeared to be blocked by NMDG⁺. For example, in the hair cell in Figure 1, the maximum steady-state current ran down 17% from the control records to the wash records. We did not study the persistent current further. A persistent NMDG⁺-sensitive component can also be seen in recordings from rat outer hair cells (Oliver et al. 1997; estimated at 7% of peak current from their Figures 1A and B).

Voltage dependence of transient inward currents. Inactivation protocols (Fig. 2B) were used to generate inactivation curves for all 167 hair cells with Na⁺ current. For 97 of these cells, activation curves were also generated from activation protocols (Fig. 2A). Conductances were
calculated from peak currents and driving forces and normalized (Fig. 2C). Most activation and inactivation curves were well fit by a single Boltzmann function (Eq. 1, Materials and Methods, Fig. 2C). Figure 2 also shows the distributions of $V_{1/2}$ and $S$ values for inactivation (Fig. 2D,E) and $V_{1/2,\text{act}}$ values (Fig. 2G) for hair cells between P0 and P4. We restricted the histograms to this age range because, as shown later (Fig. 8C), values change with age.

The Na\(^+\) currents of individual hair cells fell into two categories according to their voltage dependence of inactivation. This is shown by data from two exemplar cells (Fig. 2C) and by the clearly bimodal distribution of $V_{1/2,\text{inact}}$ values (Fig. 2D). The distribution was well fit with two Gaussian functions, which showed almost no overlap, both falling to near zero at $-81 \text{ mV}$ (arrow, Fig. 2D). This distribution suggests that most cells expressed one of two types of Na\(^+\) current with very different voltage ranges of inactivation. We will refer to currents with $V_{1/2,\text{inact}}$ values negative to $-81 \text{ mV}$ as $I_{\text{Na,1}}$ and those with $V_{1/2,\text{inact}}$ values positive or equal to $-81 \text{ mV}$ as $I_{\text{Na,2}}$.

The average values of inactivation curve parameters for cells from P0-P4 rats were: $I_{\text{Na,1}}$: $V_{1/2,\text{inact}} = -92 \pm 0.6 \text{ mV}$, $S = 6.9 \pm 0.24 \text{ mV}$, $g_{\text{max}} = 10.1 \pm 0.92 \text{ nS}$ (n = 68 cells); $I_{\text{Na,2}}$: $V_{1/2,\text{inact}} = -74 \pm 0.5 \text{ mV}$, $S = 6.0 \pm 0.24 \text{ mV}$, $g_{\text{max}} = 8.4 \pm 1.11 \text{ nS}$ (n = 38 cells). The $V_{1/2}$ values are highly significantly different ($p < 0.001$); $S$ and $g_{\text{max}}$ values are not. Curves generated from Boltzmann functions with these $V_{1/2}$ and $S$ parameters are shown in Figure 2F (thin lines).

Although the bimodal $V_{1/2,\text{inact}}$ distribution is consistent with most hair cells expressing one or the other current, the distribution of $S$ values (Fig. 2E), a measure of the width of the curves, suggests that some hair cells may have expressed both currents. For $V_{1/2,\text{inact}}$ values between $-90$ and $-75 \text{ mV}$ (double-headed arrow in Fig. 2E), inactivation curves tended to be broader than those for cells at the extremes of the distribution ($p<0.05$; one-way ANOVA). $S$ values at $-82 \text{ mV}$ were significantly broader than those between $-66 \text{ mV}$ and $-75 \text{ mV}$ and between $-87 \text{ mV}$ and $-99 \text{ mV}$. $S$ values at $-85 \text{ mV}$ were significantly broader than those between $-69 \text{ mV}$ and $-75 \text{ mV}$ and between $-90 \text{ mV}$ and $-96 \text{ mV}$ ($p<0.05$; Tukey post-hoc means comparison).
mean S values at the extremes also differed significantly (p < 0.01): S was 6.1 ± 0.15 mV for pooled $V_{1/2,\text{inact}}$ values negative to −90 mV (n = 46) and 5.3 ± 0.18 mV for pooled $V_{1/2,\text{inact}}$ values positive to −75 mV (n = 26). A plausible explanation for the broader inactivation ranges of the intermediate cells is that they expressed appreciable amounts of both currents, as illustrated for one cell in Figure 2F (open circles). Its inactivation curve was well fit either by a single Boltzmann with a large S value (8.7 mV; not shown) or by a sum of two Boltzmanns with the $V_{1/2,\text{inact}}$ and S values set at the means for $I_{\text{Na,1}}$ and $I_{\text{Na,2}}$ (thick curve).

The distributions of activation $V_{1/2}$ values for the two currents showed more overlap than did the inactivation ranges but also were consistent with two populations (Fig. 2G). Mean Boltzmann parameters for activation curves were: $I_{\text{Na,1}}$: $V_{1/2,\text{act}} = -38 ± 0.5$ mV, $S = 7.9 ± 0.16$ mV, $g_{\text{max}} = 13.1 ± 1.07$ nS (n = 54 cells); $I_{\text{Na,2}}$: $V_{1/2,\text{act}} = -31 ± 0.6$ mV, $S = 6.7 ± 0.21$ mV, $g_{\text{max}} = 10.3 ± 1.32$ nS (n = 28 cells). Again, the $V_{1/2}$ values, but not S or $g_{\text{max}}$, differ significantly (p < 0.001).

Note that $g_{\text{max}}$ values from activation curves were larger than those from inactivation curves, which were obtained for a sub-maximal depolarization (to −17 mV). Inactivation $g_{\text{max}}$ was 83 ± 2.2% of activation $g_{\text{max}}$ for $I_{\text{Na,1}}$ (n = 54) and 77 ± 3.7% for $I_{\text{Na,2}}$ (n = 28).

Some experiments were done on hair cells dissociated from the utricular macula (Figs. 1 and 5). The maculae were treated with papain, which is known to affect the voltage dependence of other channels (Armstrong and Roberts, 1988). We found that the voltage dependence of Na$^+$ current inactivation in 12 papain-dissociated hair cells (P1-P2) was consistent with two populations of Na$^+$ channels but the peak currents were smaller and inactivation ranges were shifted negatively relative to data recorded in situ. For the more negative group of dissociated cells (n = 8, P1-P2), $V_{1/2,\text{inact}} = -109 ± 2.3$ mV, 18 mV negative to the mean for $I_{\text{Na,1}}$ from the intact macula at the same ages (−91 ± 0.8 mV; n = 39; p < 0.001). The maximum current density was also 37% smaller in the dissociated cells: −78 ± 10.4 pA/pF vs. −123 ± 14.6 pA/pF; p < 0.05. For the more positive group of dissociated cells (n = 4, P1-P2) $V_{1/2,\text{inact}}$ values were significantly
more negative than in situ values for $I_{Na,2}$ over the same age range: $-82 \pm 2.0$ mV ($n = 4$) vs. $-74 \pm 1.0$ mV ($n = 11$; $p < 0.05$). Maximum current density was also lower in the dissociated cells ($-49 \pm 9.9$ pA/pF, $n = 4$, vs. $-120 \pm 39$ pA/pF, $n = 11$; $p < 0.05$). In contrast, $V_{1/2,act}$ values for both groups of dissociated hair cells did not differ significantly from those recorded in situ. Thus, for both $I_{Na,1}$ and $I_{Na,2}$, papain dissociation negatively shifted the voltage dependence of inactivation and reduced total current, but did not selectively eliminate either type of Na$^+$ current or affect the voltage dependence of activation.

Our usual external medium, Cs$^+$-SES, contained elevated Ca$^{2+}$ (5 mM), which might shift voltage dependence positively (Hille 2001). We obtained some measurements in physiological divalent levels (1.3 mM Ca$^{2+}$) for comparison. $V_{1/2,inact}$ values for $I_{Na,1}$ were 4 mV more negative ($p < 0.001$): $-96 \pm 0.9$ mV ($n = 20$) vs. $-92 \pm 0.6$ mV ($n = 68$) in 5 mM Ca$^{2+}$. For $I_{Na,2}$, however, $V_{1/2,inact}$ values were unchanged: $-75 \pm 1.3$ mV (1.3 mM Ca$^{2+}$; $n = 10$) vs. $-75 \pm 0.5$ mV (5 mM Ca$^{2+}$; $n = 48$).

*Effect of Ca$^{2+}$ current on voltage dependence.* With our solutions and protocols, Ca$^{2+}$ currents were elicited as well as Na$^+$ currents. To check for effects of the Ca$^{2+}$ current on our $V_{1/2}$ and $S$ values for the Na$^+$ current, we isolated Ca$^{2+}$ currents in two ways: by replacing Na$^+$ with NMDG$^+$ to eliminate Na$^+$ current, and by applying an activation protocol without a prepulse to inactivate $I_{Na,1}$ ($I_{Na,2}$ was not completely inactivated at the holding potential of $-67$ mV). Comparison of $V_{1/2}$ and $S$ values for total inward current and for currents with the Ca$^{2+}$ component subtracted suggests that Ca$^{2+}$ currents had little impact. For eight hair cells in the semi-intact utricular macula, there was no significant difference in $V_{1/2,act}$ ($-38 \pm 1.9$ vs. $-39 \pm 1.9$ mV), $S$ (8.4 $\pm 0.40$ vs. 7.9 $\pm 0.41$ mV) or $g_{max}$ (7.6 $\pm 1.90$ vs. 7.8 $\pm 2.10$ nS) between the records with a prepulse (Na$^+$ plus Ca$^{2+}$ current) and the difference records (prepulse – no pulse; Na$^+$ current alone), respectively. For three isolated utricular hair cells in which currents were recorded in control and NMDG$^+$ solutions, $V_{1/2,act}$ was $-35 \pm 1.5$ mV and $S$ was $8.1 \pm 0.40$ mV for the peak
total inward current vs. −35 ± 1.0 mV and 7.8 ± 0.45 mV for the peak NMDG⁺-blocked current. $G_{\text{max}}$ was significantly reduced (p < 0.05; 6.0 ± 1.17 vs. 3.9 ± 0.68 nS), probably reflecting rundown of current in the NMDG⁺ condition (see Fig. 1).

*Activation and inactivation kinetics.* Some Na⁺ currents can be distinguished by variations in kinetics: for example, TTX-insensitive currents carried by Na\(_\text{v}\)1.8 and Na\(_\text{v}\)1.9 subunits have relatively slow kinetics (Dib-Hajj et al. 2002). Both $I_{\text{Na,1}}$ and $I_{\text{Na,2}}$ had faster kinetics than Na\(_\text{v}\)1.8 or Na\(_\text{v}\)1.9 channels. To compare kinetics of activation and inactivation and of recovery from inactivation of $I_{\text{Na,1}}$ and $I_{\text{Na,2}}$, we chose hair cells well separated in the distribution shown in Figure 2D and therefore, by our analysis, likely to express mostly one or the other current: six hair cells with $I_{\text{Na,1}}$ (range of $V_{1/2,\text{inact}}$ values: −90.2 to −93.4 mV) and six with $I_{\text{Na,2}}$ (range of $V_{1/2,\text{inact}}$ values: −69.9 to −74.3 mV). We fit the time course of current decay, recorded with the usual activation protocol, with a single exponential function (Eq. 5; Fig. 2A, thick curve) and log(time constant) values were plotted against membrane potential (Fig. 3A). For both $I_{\text{Na,1}}$ and $I_{\text{Na,2}}$, the inactivation time constant and the time to peak, a measure of the speed of activation, became faster with more depolarized steps. Kinetic differences between the two currents were small but significant (two-way ANOVA on logarithmically transformed values of Fig. 3A,B; p < 0.001). The difference did not reflect an average difference in uncompensated series resistance for the two groups, which produced similar average maximum voltage errors (the product of residual series resistance and maximum currents): 4.4 ± 0.89 mV for $I_{\text{Na,1}}$ (range 2.9 - 7.1, n = 6) and 4.5 ± 1.29 mV for $I_{\text{Na,2}}$ (range 2.4 -10.4, n = 6). The $\tau_{\text{inact}}$ values for $I_{\text{Na,2}}$ are slightly slower than those for a similar Na⁺ current in mouse inner hair cells, reflecting at least in part the higher temperature of the inner hair cell recordings (34 - 37°C; Marcotti et al. 2003). Times to peak for both $I_{\text{Na,1}}$ and $I_{\text{Na,2}}$ were shorter than the time to peak for $I_{\text{Na}}$ in chick crista cells obtained with a similar protocol at room temperature (Masetto et al. 2003; −120 mV prepulse): ~400 µs vs. ~600 µs at −10 mV.
For currents that are inactivated at resting potential, the time course of inactivation removal at hyperpolarized potentials is functionally relevant. We investigated the time required for a step to $-137$ mV to remove the inactivation accumulated at the holding potential ($-67$ mV) in 12 hair cells with $I_{Na,1}$ (Fig. 3C) and in five hair cells with $I_{Na,2}$ (Fig. 3D). In six hair cells with $I_{Na,1}$ and four hair cells with $I_{Na,2}$, the recovery of the peak response to a step to $-17$ mV was best fit with a double exponential function (dashed fits in Fig. 3C, D). Mean time constants were $1.1 \pm 0.20$ ms and $12.6 \pm 2.52$ ms for $I_{Na,1}$ and $0.5 \pm 0.05$ ms and $15.4 \pm 3.83$ ms for $I_{Na,2}$. The fast time constants for the two types of current differed significantly ($p < 0.05$). In both cases the size of the fast component exceeded the size of the slower component by at least two orders of magnitude. The slow second time constant may represent recovery from a more inactivated state.

In the other hair cells (six with $I_{Na,1}$ and one with $I_{Na,2}$), recovery followed a single exponential time course with time constants of $1.8 \pm 0.25$ ms ($I_{Na,1}$) and $1.9$ ms ($I_{Na,2}$). The dominant fast constants of recovery are consistent with data from type II hair cells of the chick crista (Masetto et al. 2003), after extrapolation for our more negative conditioning voltage.

**Evoked spiking in current clamp recordings.** Voltage-gated Na$^+$ channels are usually assumed to play a role in action potential firing, or spiking. We looked for spiking in current clamp recordings made with K$^+$-based, rather than Cs$^+$-based, solutions (K$^+$-SES, K$^+$-SIS) from hair cells in the semi-intact utricular macula (10 cells with $I_{Na,1}$, mean input resistance $615 \pm 117$ M$\Omega$, zero-current potential $-54 \pm 4.1$ mV; 8 cells with $I_{Na,2}$, mean input resistance $576 \pm 45.8$ M$\Omega$, zero-current potential $-55 \pm 1.9$ mV). We never saw spontaneous spiking and depolarizing current steps usually failed to evoke spikes unless preceded by a hyperpolarizing current step. In this regard, our data resemble previous recordings from immature hair cells of rat and mouse utricular macula (Chabbert et al. 2003; Géléoc et al. 2004), immature rat outer hair cells (Oliver et al. 1997), and more mature chick crista hair cells (Masetto et al. 2003).
Figure 4 shows, for a cell with $I_{Na,2}$ (Fig. 4A) and a cell with $I_{Na,1}$ (Fig. 4B), how the size and time course of the spike-like event at the onset of a large depolarizing current step depended on pre-step voltage. (We consider these events to be spike-like because shoulders in the depolarizing phase (see arrows) indicate a threshold for regenerative current.) In three cells with $I_{Na,2}$, including the cell in Figure 4A, the drop in peak potential with depolarization of the pre-step interval (Fig. 4C) strongly overlapped the $I_{Na,2}$ inactivation range ($V_{1/2}$ values of $-68 \pm 2.0 \text{ mV}$ vs. $-76 \pm 1.3 \text{ mV}$, respectively). Thus, the fall-off in peak potential with pre-step depolarization may be attributable to $I_{Na,2}$ inactivation. Chabbert et al. (2003) blocked similar spikes in a rat utricular hair cell with $I_{Na,2}$ with 100 nM TTX. Thus, Na$^+$ channel openings for $I_{Na,2}$ might boost the spikes elicited from potentials negative to $-60 \text{ mV}$. In contrast, when the same current protocol was applied to three cells with $I_{Na,1}$, the peak potential did not change over the inactivation range of $I_{Na,1}$ (Fig. 4B,D), suggesting that $I_{Na,1}$ did not determine the peaks. The fall-off in peak potential for pre-step potentials positive to $-65 \text{ mV}$ might reflect the activation of outwardly rectifying K$^+$ channels, which can also be seen in the current record (arrows, Figs. 4C,D).

The two Na$^+$ currents had different tetrodotoxin sensitivities. TTX separates Na$^+$ currents into those that are TTX-sensitive, with $K_D$’s in the low nanomolar range, and those that are TTX-insensitive, with $K_D$’s in the high nanomolar or micromolar range. We tested the TTX sensitivity of the Na$^+$ currents in hair cells dissociated from the rat utricular macula. For five hair cells with $I_{Na,1}, 500 \text{ nM TTX blocked Na current (}I_{Na,1}\text{) by just }53 \pm 4.8\% (\text{Fig. 5A). If the underlying channels were a uniform population, this block is consistent with a dissociation constant, }K_D, \text{ in the TTX-insensitive range (440 nM, calculated assuming a 1:1 ratio of TTX molecules to channels; Hille 2001). Although the presence of a small contaminating Ca$^{2+}$ current could reduce the apparent block, note that in the example shown in Figure 5A, the current in TTX had no sustained component – i.e., no Ca$^{2+}$ current – and clearly had a transient component, i.e., Na$^+$ current. Thus, there is no doubt that the cell had a TTX-insensitive Na$^+$ current.
For three hair cells with $I_{Na,2}$, a much lower concentration of TTX (50 nM) blocked the Na current ($I_{Na,2}$) by 76 ± 8.1% (Fig. 5B). Under the same assumptions, such a block is consistent with a TTX-sensitive $K_D$ of 16 nM. In the example in Figure 5B, the presence of a TTX-insensitive sustained component (see data in TTX) is consistent with a $Ca^{2+}$ current. Any $Ca^{2+}$ current contamination of the peak current, however, would lead us to underestimate the TTX sensitivity of the Na$^+$ current.

In summary, most rat utricular hair cells between P0 and P4 expressed one of two Na$^+$ currents with different voltage dependence and sensitivity to block by TTX. $I_{Na,1}$ had TTX sensitivity and kinetics comparable to those of cardiac Na$^+$ currents. The cardiac Na$^+$ current also has a more negative voltage dependence than most brain Na$^+$ currents (Discussion). These similarities suggest that $I_{Na,1}$ might be carried by channels similar to those responsible for the cardiac Na$^+$ current. $I_{Na,2}$ had TTX sensitivity, kinetics and voltage dependence in the range of several neuronal channels. In the next sections, we show how expression of the two Na$^+$ currents depended on hair cell location in the utricular macula, hair cell type, and postnatal age.

Expression of the two Na$^+$ currents varied with location in the immature epithelium

The utricular macula has two regions, the striola and extrastriola, with distinct morphology and afferent physiology. We examined how the two Na$^+$ currents were distributed relative to these regions (Fig. 6A). Because of developmental changes (see next section), we included only cells from ages P0 to P4 ($n = 110$) in the histogram. In this period, all but four cells expressed Na$^+$ current. Most (90%, 45/50) striolar hair cells expressed $I_{Na,1}$; the remaining five cells expressed $I_{Na,2}$. In contrast, just over half (33) of 60 extrastriolar cells expressed $I_{Na,2}$, with 23 cells expressing $I_{Na,1}$ and four cells lacking any Na$^+$ current. Breaking the extrastriolar data of Figure 6A into values from the lateral and medial zones (LES and MES, Fig. 6B) revealed that nearly all extrastriolar cells with $I_{Na,1}$ were from the MES. The lateral edge of the extrastriola, furthest from the point of entry of the utricular nerve branch, was almost devoid of $I_{Na,1}$. The difference in our
LES and MES data might alternatively reflect a peripheral vs. central difference, since MES cells were taken mostly from the central part of this zone while LES cells are perforce near the peripheral edge of the epithelium (see inset in Fig. 6A).

Based on whole-cell capacitance ($C_m$) values, which are proportional to cell surface area, cells at all ages expressing $I_{Na,1}$ had 16% more surface area than cells expressing $I_{Na,2}$: $7.0 \pm 0.13$ pF ($n = 122$) vs. $6.0 \pm 0.23$ pF ($n = 45; p < 0.001$). Similar values were obtained in the smaller P0-P4 data set and when striola and extrastriola were considered separately. The size difference might reflect a difference in cell type. Identified type I cells ($7.2 \pm 0.26$ pF, $n = 26$) were larger than type II cells ($6.1 \pm 0.31$ pF, $n = 30; p < 0.01$) between P9 (the youngest age at which type II cells can be confidently identified) and P22. Some of the capacitance difference might reflect different hair bundle surface areas. Alternatively, cells with $I_{Na,2}$ might be smaller if they are at an earlier developmental stage. This seems unlikely, however, as we saw no change in $C_m$ with age (P0-P22), either overall or within groups (striola, extrastriola, $I_{Na,1}$ and $I_{Na,2}$).

**Type I cells expressed $I_{Na,1}$**

Calyces form and expand in the first postnatal week in the rat utricle. We were able to classify a hair cell as type I (mature or immature) if a partial or full calyx was visible around it (see Materials and Methods). When no calyx was visible, a hair cell could be an immature hair cell of either type. Most calyces and therefore most identified hair cells were in the striola. All 50 hair cells with calyces (39 striolar, 11 extrastriolar) expressed $I_{Na,1}$.

We classified 30 cells as type II based on their lack of calyces at P9 or older; even this group may include some type I hair cells that have yet to receive a calyx ending. Nearly half (14) of these ‘type II’ cells, approximately evenly spread across zones (striola, LES, MES), lacked any $Na^+$ current. Of the remaining 16 type II cells, 15 expressed $I_{Na,1}$ (again, evenly distributed across zones); only one cell expressed $I_{Na,2}$. Note that we classify cells as type II only after the first postnatal week, when $I_{Na,2}$ had largely disappeared from the macula (see next section). Many
type II cells at earlier stages, here considered unclassified, must express I_{Na,2}: unclassified hair cells with I_{Na,2} (all from P0-P8) were unevenly distributed across zones: 6/35 (17%) of unclassified striolar cells and 38/66 (58%) of unclassified extrastriolar cells had I_{Na,2}.

In the mature rat utricular macula, ~50% of hair cells in both zones are type I (Desai et al. 2005b). Similar percentages are likely to hold in the immature epithelium, given evidence that vestibular hair cells in mice are born (have undergone their final division) by P3 (Ruben, 1967). If so, then our data suggest that from P0-P22 all type I hair cells and a significant fraction of immature type II cells in the striola express I_{Na,1} (see Discussion). It is possible that, at least during the first postnatal week, I_{Na,2} is expressed only by immature type II cells. The next section shows dramatic changes after the first postnatal week.

Expression of the two Na\(^+\) currents varied with postnatal age

Cell size, as measured by \(C_m\), did not change significantly from P0-P22 (linear regression analysis yielded a line with slope 20 fF/day, \(n = 167, r^2 = 0.005\)). Peak-current densities (peak current/\(C_m\)) for I_{Na,1} and I_{Na,2} varied with age and region. To calculate current densities, we took peak currents from the Boltzmann fits of inactivation data because we recorded inactivation protocols for all cells. Recall that these values were obtained for a test step to a sub-maximal depolarization (~17 mV) and thus underestimate peak current density by about 20%. Figure 7 shows, for each current and each zone, how current density varied with age (A, B) and how incidence (% cells expressing a current) varied with age (C, D). Both currents were largest and detected most frequently during the first postnatal week.

I_{Na,1} density in striolar hair cells remained fairly constant from P0 to P9 (Fig. 7A). By P15, however, I_{Na,1} had fallen to ~50 pA/pF, one third of its average density between P1 and P4. In the extrastriola (LES + MES), I_{Na,1} density fell by a similar amount but earlier (after P4): the average I_{Na,1} density from P6 to P22 was 31% of that from P0 to P4 (Fig. 7A). According to a
two-way ANOVA on the data contributing to Figure 7A, striolar $\text{I}_{\text{Na,1}}$ density exceeded extrastriolar $\text{I}_{\text{Na,1}}$ density ($p<0.00001$) and $\text{I}_{\text{Na,1}}$ density varied with age ($p<0.005$; a post-hoc Tukey means test found that density at P2 significantly exceeded density at P20).

Mean $\text{I}_{\text{Na,2}}$ density in the extrastriola plummeted after P3 (Fig. 7B). From P0-P3, one-third of extrastriolar cells with $\text{I}_{\text{Na,2}}$ had densities $\geq 300$ pA/pF, while the rest had densities below 180 pA/pF. After P3, the larger current densities disappeared, reducing both the mean and the variance. In the striola, only six hair cells had $\text{I}_{\text{Na,2}}$; their current densities seemed in line with the extrastriolar data (Fig. 7B).

With respect to incidence, $\text{I}_{\text{Na,1}}$ always dominated in the striola (Fig. 7C). $\text{I}_{\text{Na,2}}$ dominated in the extrastriola in the first week (59% of cells; 19/24 LES cells, 21/46 MES cells; Fig. 7D). From P13 onwards, 63% of all cells (13/18 striolar, 3/8 LES, 8/12 MES) expressed $\text{I}_{\text{Na,1}}$. The remaining 37% with no detectable Na$^+$ current matches the percentage of all cells in the first postnatal week that either expressed $\text{I}_{\text{Na,2}}$ or had no current (48/129). This raises the possibility that they are the same cell populations; i.e., that cells dominated by $\text{I}_{\text{Na,2}}$ early on did not convert to $\text{I}_{\text{Na,1}}$ later.

For $\text{I}_{\text{Na,1}}$, we had enough data to separate the current density data (Fig. 7A) according to cell type (Fig. 8A,B). This shows that the zonal difference in Figure 7A (larger currents in the striola than the extrastriola at all ages) holds for type I hair cells (Fig. 8A), and for unclassified and type II hair cells (Fig. 8B), considered separately. That is, there are no significant differences between the current densities of $\text{I}_{\text{Na,1}}$ in type I cells and in other cells (unclassified and type II cells pooled), as shown by a two-way ANOVA ($p = 0.76$) and post-hoc Tukey means comparison ($p>0.05$). Cells with low $\text{I}_{\text{Na,1}}$ density ($<60$ pA/pF) were seen at all ages, but densities $>200$ pA/pF were only recorded in the first week. The wide range of current densities in the first postnatal week may reflect hair cell differentiation occurring at different rates even within a single zone, as observed in the perinatal mouse utricular macula (Denman-Johnson and Forge 1999; Géléoc et al. 2004). Figure 8C shows that $V_{1/2,\text{inact}}$ values for both currents hyperpolarized by about 1 mV/day (Fig. 8C).
In summary, almost all striolar cells expressed \( I_{\text{Na},1} \), no matter what age, but the mean current density dropped from the first to third weeks. Many extrastriolar cells expressed \( I_{\text{Na},2} \) in the first week. The overall numbers are consistent with these being immature type II cells. In addition, the numbers suggest that the population lacking all \( \text{Na}^+ \) current in the third week could be drawn from the population with \( I_{\text{Na},2} \) in the first week.

**\( \text{NaV1.5 is a candidate} \ \alpha \ \text{subunit for} \ \text{I}_{\text{Na},1} \)**

Our physiological data support the existence of two distinct \( \text{Na}^+ \) currents that are differently expressed across region, hair cell type and age in the early postnatal rat utricular macula. To screen for molecular candidates for the currents, we used the RT-PCR method to probe utricular maculae and semicircular canal cristae at P1 and P21, and single utricular hair cells at P1 for \( \text{Na}^+ \) channel mRNA, and immunocytochemistry to look for localization of \( \text{Na}^+ \) channel protein.

**RT-PCR.** We used primers corresponding to each of the nine pore-forming \( \text{Na}^+ \) channel \( \alpha \) subunits (Fig. 9A, B) and each of the four accessory (\( \beta \)) subunits (Fig. 9B). The thermolysin-treated epithelia contain hair cells, supporting cells and nerve terminals. Figure 9C shows the incidence of positive results for each primer set in each tissue at each age; the numbers of experiments are shown above the bars. The TTX sensitivities of the two currents narrow the field of possible \( \alpha \)-subunit candidates for each type.

**\( \alpha \)-subunit candidates for \( I_{\text{Na},1} \).** Known TTX-insensitive subunits are \( \text{NaV1.5}, \ \text{NaV1.8} \) and \( \text{NaV1.9} \). As discussed in the context of Figure 5, the \( K_D \) for TTX of \( \text{NaV1.5} \) is the best match with the TTX sensitivity of \( I_{\text{Na},1} \). Our RT-PCR results offer further support that \( I_{\text{Na},1} \) is carried by \( \text{NaV1.5} \) channels: PCR products corresponding to \( \text{NaV1.5} \), but not \( \text{NaV1.8} \) or 1.9, were obtained from utricular maculae at both P1 and P21 (Fig. 9B, C). Mechaly et al. (2005) also saw no \( \text{NaV1.8} \) when they screened P1 rat utricles for various \( \text{NaV} \) subunits. We obtained a light positive band for \( \text{NaV1.8} \) from one of six experiments on P1 cristae (Fig. 9C).
**Single-cell RT-PCR.** PCR products from thermolysin-treated epithelia cannot categorically confirm the presence of subunit-specific message in hair cells. Thus, we collected 11 individual hair cells from an epithelium and performed single-cell RT-PCR (Fig. 9E). All 11 cells were positive for β-actin. In 3 cells (2 striolar [lanes 1 and 8 in gel] and 1 extrastriolar [lane 4]), PCR product was obtained for both Na\(_V\)1.5 and Na\(_V\)1.2. Cell 6 was positive for Na\(_V\)1.2 alone.

**Immunocytochemistry.** We examined utricular epithelia for Na\(_V\)1.5-, Na\(_V\)1.2- and Na\(_V\)1.6-like immunoreactivity at various stages between P1 and adult (Fig. 10). We also applied an antibody against calretinin (Figs. 11 and 12), which provides a useful regional marker: calretinin is selectively expressed by striolar afferents that only contact type I hair cells (‘calyx afferents’). Because calyx afferents are largely confined to the striolar zones of the utricular and saccular maculae and the central zones of the cristae, respectively (Desmadryl and Dechesne 1992; Leonard and Kevetter 2002; Desai et al. 2005a,b), staining with calretinin helps demarcate those regions. In rats and mice, immature hair cells (Zheng and Gao 1997) and 70% of mature type II hair cells are also immunoreactive for calretinin (Desai et al. 2005b).

Cytoplasmic staining of hair cells was evident in the first few postnatal days for all three Na\(^+\) channel antibodies (P0-P1 and P3 shown) in the utricular macula (Figs. 10, 11), contemporaneous with the largest Na\(^+\) currents. Thereafter, hair cell staining faded while staining rose in afferent ending terminals and fibers (Na\(_V\)1.5; Fig. 10), and in supporting cells (Na\(_V\)1.2 and 1.6; Fig. 10).

**Na\(_V\)1.5-like immunoreactivity.** Na\(_V\)1.5-like immunoreactivity was particularly strong for some hair cells at P1 and P3 (double arrowheads, Figs. 10, 11A). Such cells might correspond to the minority (11/68 cells, P0-P4) with especially large peak \(I_{Na,1}\) (>1.5 nA, corresponding to 500-1000 channels for single-channel conductances in the range of 20-40 pS; Benndorf 1994; Fozzard and Hanck 1996; Wartenberg et al. 2001). Other hair cells showed light somatic staining (single arrowheads, Fig. 10: P1, P3). No distinction was obvious between zones. The regional variation in Na\(^+\) current expression (Fig. 6) had led us to anticipate Na\(_V\)1.5 immunoreactivity in fewer extrastriolar cells (one-third vs. 90% of striolar cells) between P0 and P4, especially in the lateral
extrastriola. If $I_{\text{Na},1}$ is carried by $\text{Na}_V1.5$ channels, then the patterns of currents, single-cell expression and immunoreactivity suggest that co-expression of $I_{\text{Na},2}$ masks a small $I_{\text{Na},1}$ in many extrastriolar hair cells in much of the first postnatal week. By P6-P8, the strongest $\text{Na}_V1.5$ staining within the epithelium appeared to be associated with the calyx membrane facing the hair cell (the ‘inner-face’ membrane; see arrows in Fig. 10, P6; Fig. 11B). Staining was still evident in hair cells even up to P21, including type I cells (arrowheads, Figs. 10; 11B). In the P21 whole-mount of the utricular macula (Fig. 12), punctate cytoplasmic staining is seen in many hair cells. There are also holes corresponding to $\text{Na}_V1.5$-negative hair cells in both zones (asterisks). Calretinin-positive (green) hair cells, which are usually type II cells (Desai et al. 2005b), show a range of $\text{Na}_V1.5$-like (red) immunoreactivity, from none to bright staining (yellow spots, open arrowheads, Fig. 12). Type I hair cells are surrounded by rings of label, which are bright and mostly continuous in the striola (Fig. 12A) and somewhat dimmer and more punctate in the extrastriola (Fig. 12B). Much of this staining appears to be in the calyx inner face membrane: in the striola, where many calyces are immunoreactive for calretinin, yellow bands are seen where the fluorescein label (green) for the cytoplasmic calretinin overlaps with the membrane-associated rhodamine label (red) for $\text{Na}_V1.5$ (arrowheads, Fig. 12A). The resolution of confocal immunostaining prevents us from being definitive as to the location of this staining. Electron micrographs of type I/calyx afferents, however, confirm the postsynaptic staining for $\text{Na}_V1.5$ antibodies (S. Gaboyard and A. Lysakowski, unpublished observations). Staining for $\text{Na}_V1.5$ is particularly strong in bands between type I hair cells within a complex calyx (arrows, Fig. 10, P6; Fig. 12A), reflecting the proximity of two sets of inner-face and hair cell membranes, with no interposed supporting cells. Even immature partial calyces stained, as early as P1 (Fig. 11A,, thin arrow). Strong staining was also seen at all ages in afferent fibers below the macula (Fig. 10 (left) and Fig. 11A,B, filled arrows).

In summary, $\text{Na}_V1.5$-like immunostaining was present in type I and unclassified and type II cells, and was strongest in some hair cells in the first postnatal week. These observations are
consistent with whole-cell recordings of $I_{Na,1}$. Zonal differences in hair-cell (as opposed to afferent) immunoreactivity were not obvious (Fig. 10). Although the mean $I_{Na,1}$ current densities in the two zones clearly differed (Fig. 7A), large scatter in individual current densities (see large standard errors) suggests that zonal differences in immunoreactivity might be hard to detect. Alternatively, $I_{Na,1}$ might be expressed at a low level even in hair cells that also express $I_{Na,2}$, which would be difficult to detect electrophysiologically. There was a high level of NaV1.5-like-immunoreactivity in calyx inner-face membranes and within afferent fibers.

**Multiple candidates for $I_{Na,2}$**

*RT-PCR.* Of six TTX-sensitive $\alpha$ subunits, PCR products for five were obtained consistently at P1 in all tissues: NaV1.1, 1.2, 1.4, 1.6 and 1.7 (Figs. 9A, C). In an RT-PCR screen of P1 rat utricles, Mechaly et al. (2005) obtained PCR products for all TTX-sensitive subunits tested (NaV1.1, 1.2, 1.3, 1.6 and 1.7). NaV1.3 subunits may be present in vestibular epithelia at lower levels than other Na$^+$ channel $\alpha$ subunits. We detected NaV1.3 when we did two rounds of amplification (total 80 cycles; data not shown; 2 utricular epithelia). In a single-cell RT-PCR analysis of P1-P3 rat utricular hair cells, Chabbert et al. (2003) saw NaV1.3 expression in three of thirteen cells compared to seven cells with NaV1.2 and ten cells with NaV1.6.

The NaV1.6 primers used by Chabbert et al. (2003) and by us for the data in Figure 9A did not differentiate three splice variants involving exon 18 (Plummer et al. 1997): an “adult” variant (18A) which is functional; a “neonatal” variant (18N), which is truncated and presumed non-functional; and a non-functional variant with exon 18 deleted ($\Delta$18) that is presumably never made. With primers bracketing exon 18, Mechaly et al. (2005) found only the 18N and $\Delta$18 forms in P1 and P10 rat utricles. We confirmed the result in P1 utricular epithelia and got similar results in P1 cristae (Fig. 9D). The P1 ganglion served as a control: in contrast to the epithelia, P1 ganglia expressed both neonatal and adult forms (Fig. 9D). Thus, NaV1.6 is eliminated as a
candidate for $I_{Na,2}$ in vestibular hair cells, but may play a role in $Na^+$ currents in the afferent neurons.

At P21, there were two changes in TTX-sensitive $\alpha$ subunits (Figs. 9A, C): $Na_V1.4$ mRNA was no longer detected in any inner ear tissue and $Na_V1.7$ mRNA was no longer detected in the utricular epithelia (0/3 experiments) or reliably in the cristae (detected in 1 of 3 experiments). Mechaly et al. (2005) also did RT-PCR for $Na_V1.7$ on rat utricles and saw reduced expression at P21 relative to P1.

PCR products were obtained for all four known $\beta$ subunits in all tissues at P1 (Figs. 9B, C). At P21, however, $Na_V\beta4$ was detected in just 1 of 3 tests in the utricular macula, and $Na_V\beta2$ was no longer detected in the cristae (Fig. 9D).

**$Na_V1.2$ and $Na_V1.6$ immunohistochemistry.** $Na_V1.2$ and $Na_V1.6$ were the most prevalent of the TTX-sensitive $\alpha$ subunits tested in the single-cell RT-PCR analysis by Chabbert et al. (2003). The $Na_V1.6$ antibody, which is made against a region of the protein held in common by the exon 18 splice variants, produced some of the brightest staining, described in more detail below. This is consistent with the high incidence of $Na_V1.6$ products in single-cell RT-PCR (Chabbert et al. 2003). mRNA corresponding to the truncated $Na_V1.6$ subunits is highly expressed in the early embryonic mouse brain and in adult non-neuronal tissues; comparable isoforms exist in humans and fish (Plummer et al. 1997). Although these forms cannot form channels, their evolutionary conservation and widespread expression suggest that they serve some function (Plummer et al. 1997). Our immunocytochemistry on vestibular epithelia suggests that significant amounts of the truncated, two-domain, 18N protein is made by immature hair cells and by supporting cells at all ages.

Figure 10 show antibody labeling for $Na_V1.2$ and $Na_V1.6$ between P1 and P21 in the utricular macula. From P0 to P8, hair cells showed cytoplasmic staining with both antibodies. Hair cell staining became less intense at older ages. From P0-P8, $Na_V1.6$-like immunoreactivity included
bright spots in hair cells, below the cuticular plate (Figs. 10, 11D, open arrowheads). One possibility is that the spots are accumulations of truncated Na\textsubscript{V}1.6 proteins made from the 18N splice variant. The striola, where most cells were classified as expressing I\textsubscript{Na,1} (Fig. 7C), stained well for Na\textsubscript{V}1.2 from P0-P3 (double arrowheads, Fig. 11C). It is possible that I\textsubscript{Na,1} masked a smaller I\textsubscript{Na,2} in these cells. By P21, hair cell staining had fallen off for both Na\textsubscript{V}1.2 and Na\textsubscript{V}1.6 antibodies (Fig. 10).

Both antibodies stained supporting cells within the macula (the sensory part of the epithelium). From P0 to P8, the apical surfaces of supporting cells within the utricular macula stained strongly with Na\textsubscript{V}1.2 antibody; this staining is shown at high magnification in Figure 11C (open arrow). Chabbert et al. (2003), using a different Na\textsubscript{V}1.2 antibody, also saw strong staining of the apical surfaces of supporting cells in the rat utricular macula.

In summary, RT-PCR screening suggests multiple candidate \(\alpha\) subunits for I\textsubscript{Na,2}. All known TTX-sensitive subunits were detected, although Na\textsubscript{V}1.1, and particularly Na\textsubscript{V}1.3, had comparatively low incidences (Fig. 9C). Na\textsubscript{V}1.4 and Na\textsubscript{V}1.7 PCR products were detected at P1 but not at P21; Na\textsubscript{V}\(\beta\)4 mRNA expression fell off from P1 to P21 in the utricular macula. These changes suggest candidates for the loss of I\textsubscript{Na,2} in the same period. In addition, the cellular localization of subunits may undergo developmental changes that cause loss of current but that are not detectable by RT-PCR on whole epithelia. For example, Chabbert et al. (2003) reported a developmental shift in the localization of Na\textsubscript{V}1.2-like immunoreactivity from hair cells to supporting cells. We also saw loss of Na\textsubscript{V}1.2-like immunoreactivity from hair cells together with increasing or persistent immunoreactivity in supporting cells.

**Discussion**

**Two Na\textsuperscript{+} currents in hair cells**

Expression of two Na\textsuperscript{+} currents with different TTX sensitivities and voltage dependence can account for the large variance in Na\textsuperscript{+} current properties recorded by different investigators in
immature rodent inner ear epithelia. $I_{\text{Na},1}$ of rat utricular hair cells resembles the current reported in immature mouse utricular hair cells by Rüscher and Eatock (1997) and Géléoc et al. (2004) and in rat cochlear outer hair cells by Oliver et al. (1997). $I_{\text{Na},2}$ resembles the TTX-sensitive current reported in immature rat utricular hair cells by Chabbert et al. (2003) and Lennan et al. (1999) and in immature mouse cochlear inner hair cells by Marcotti et al. (2003).

Why did previous studies on rodent utricular epithelia detect just one current? The answer probably involves selective sampling from particular cell types, regions or ages, and/or pooling of data from the two Na⁺ current populations. According to our sample, if you record from the striola, you will find little evidence for $I_{\text{Na},2}$, while if you record from immature type II cells, you may select for $I_{\text{Na},2}$. Rüscher and Eatock (1997) and Géléoc et al. (2004) selectively recorded from the “central” zone of the embryonic mouse utricle, which would likely include striola and some medial extrastriola. Chabbert et al. (2003) selectively recorded from the extrastriola (personal communication) and therefore may have mostly recorded $I_{\text{Na},2}$.

Rüscher and Eatock (1997) and Chabbert et al. (2003) reported that type I hair cells did not express Na⁺ current; we claim that all identified type I cells expressed Na⁺ current. This discrepancy may reflect different classification schemes. We identified hair cells as type I by their calyces. Rüscher and Eatock (1996) classified cells according to whether they expressed the type I-specific K⁺ conductance, $g_{K,L}$. In dissociated rat utricular hair cells, $g_{K,L}$ is not evident as a distinctive negatively activating conductance until P7 (Hurley et al. 2006), a week or more after its appearance in the mouse (Géléoc et al. 2004). In both mouse and rat utricles, $I_{\text{Na},1}$ amplitude declines as $g_{K,L}$ grows. Thus, the largest Na⁺ currents in rat utricular type I cells, defined by the presence of the calyx, occurred before they acquired $g_{K,L}$. The smaller currents of cells that have acquired $g_{K,L}$ would be hard to detect when $g_{K,L}$ is incompletely or not blocked, as in the experiments of Rüscher and Eatock (1997).

A third Na⁺ current type, TTX-sensitive (like $I_{\text{Na},2}$) but negatively-inactivating (like $I_{\text{Na},1}$), is reported from hair cells of diverse juvenile and mature hair cell organs (Evans and Fuchs 1987;
Sugihara and Furukawa 1989; Witt et al. 1994; Masetto et al. 2003). The channel composition may change with maturation, or, since most of these are non-mammals, differ between vertebrate classes. In some cases, it is possible that the third current is a mixture of $I_{\text{Na},1}$ and $I_{\text{Na},2}$. Data from Masetto et al. (2003) hint at heterogeneous $I_{\text{Na}}$ expression in chick crista hair cells. The reported $\text{Cd}^{2+}$ sensitivity of the Na$^+$ current suggests that a component was TTX-insensitive (Favre et al. 1995; Wooltorton et al. in press). Also, a $-80 \text{ mV}$ prepulse relieved Na current inactivation in three of five cells, but a more negative prepulse was required in two cells, consistent with $I_{\text{Na},2}$ and $I_{\text{Na},1}$, respectively.

**Na$^+$ channel subunit composition**

For $I_{\text{Na},1}$, the TTX sensitivity points to Na$\gamma$1.5 subunits. From our data, we estimated the $K_D$ as 440 nM, which is in the range estimated for the very negatively inactivating Na$^+$ currents of mouse utricular hair cells (348 nM, Rüsch and Eatock 1997) and rat outer hair cells (474 nM, Oliver et al. 1997), and for cardiac Na$^+$ currents (150 nM – 2 $\mu$M; e.g., Brown et al. 1981; Krafte et al. 1991; Arreola et al. 1993; White et al. 1993; Yoshida 1994). The TTX-insensitive Na$^+$ currents of dorsal root ganglion neurons have even higher $K_D$’s (30 $\mu$M to >100 $\mu$M; Akopian et al. 1996; Sangameswaran et al. 1996; Rabet et al. 1998; Cummins et al. 1999; Rugiero et al. 2003). Among TTX-insensitive subunits, the voltage dependence and kinetics of $I_{\text{Na},1}$ match those of the cardiac channel (Na$\gamma$1.5) better than those of the peripheral nervous system (PNS) channels, Na$\gamma$1.8 or Na$\gamma$1.9. $V_{1/2,\text{inact}}$ ranges from $-81$ to $-106 \text{ mV}$ in cardiac channels (O’Leary 1998, Yagi et al. 2002, Fearon and Brown 2004, Valdivia et al. 2005), but from $-34$ to $-55 \text{ mV}$ in PNS channels (Cummins et al. 1999; Renganathan et al. 2002). Inactivation time constants at 0 mV were 200 $\mu$s in $I_{\text{Na},1}$ (Fig. 3A), closer to the values for Na$\gamma$1.5 channels (0.6 - 1 ms; Gu et al. 1997, O’Leary 1998, Sheets and Hanck 1999) than for Na$\gamma$1.8 or Na$\gamma$1.9 channels (3 - 6 ms; Renganathan et al. 2002). Furthermore, mRNA for Na$\gamma$1.5, but not for Na$\gamma$1.8 and Na$\gamma$1.9, was
detected in the utricular macula, and staining with an antibody to Na\textsubscript{V}1.5 supported its expression by hair cells. Mechaly et al. (2005) also showed that Na\textsubscript{V}1.8 was not detected with RT-PCR in P1 rat utricles.

\(I_{\text{Na,2}}\) may flow through several kinds of Na\textsuperscript{+} channels. We estimated a \(K_D\) of 16 nM for \(I_{\text{Na,2}}\), which falls in the range of values for diverse TTX-sensitive Na\textsuperscript{+} currents in other cell types (1 – 25 nM; Noda et al. 1986; Sangameswaran et al. 1997; Dietrich et al. 1998; Smith and Goldin 1998; Smith et al. 1998; Chen et al. 2000; Moran et al. 2003). mRNA for Na\textsubscript{V}1.1, Na\textsubscript{V}1.2, Na\textsubscript{V}1.3, Na\textsubscript{V}1.6 and Na\textsubscript{V}1.7 has been detected previously in P1 rat utricles (Chabbert et al. 2003; Mechaly et al. 2005) and our RT-PCR results generally agreed with these findings. Two functional TTX-sensitive \(\alpha\) subunits undergo developmental changes at the mRNA level (Na\textsubscript{V}1.4 and Na\textsubscript{V}1.7; Fig. 9A, see also Mechaly et al. 2005 for Na\textsubscript{V}1.7) or protein level (Na\textsubscript{V}1.2; Fig.10) that might contribute to the loss of \(I_{\text{Na,2}}\). Na\textsubscript{V}1.7 provides the closest fit to the biophysical and pharmacological properties of \(I_{\text{Na,2}}\) (Marcotti et al. 2003). But immunohistochemical and single-cell RT-PCR data (present study and Chabbert et al. 2003) also suggest that Na\textsubscript{V}1.2 subunits contribute to \(I_{\text{Na,2}}\). Our findings further suggest that differences in subunit expression between cells with \(I_{\text{Na,1}}\) and \(I_{\text{Na,2}}\) may be quantitative rather than qualitative, given the lack of regional variation in Na\textsubscript{V}1.5 and Na\textsubscript{V}1.2 immunohistochemistry and the co-expression of Na\textsubscript{V}1.5 and Na\textsubscript{V}1.2 mRNA in some single cells.

mRNA for the four known \(\beta\) subunits was detected at P1 in rat vestibular epithelia. Na\textsubscript{V}1.5 subunits co-express with \(\beta1\) and \(\beta2\) subunits at the Z-lines of cardiomyocytes (Malhotra et al. 2001). Adding \(\beta\) subunits to expression systems may speed up kinetics (Smith et al. 1998) and may shift the \(\alpha\) subunits’ inactivation range positively (Fahmi et al. 2001), negatively (Isom et al. 1992; Smith and Goldin 1998; Smith et al. 1998), or not at all (Smith and Goldin 1998; Yu et al. 2003). Changing expression of \(\beta\) subunits, such as the loss of \(\beta4\) from the utricular epithelium between P1 and P21 (Fig. 9B), might contribute to the gradual hyperpolarization of the
inactivation range (~1 mV/day; Fig. 8C). In type I cells, this gradual shift might be tracking a gradual hyperpolarization of resting potential over the same period, as K⁺ channels activate more and more negatively (Hurley et al. 2006).

β subunits may also enhance α-subunit trafficking to the hair cell membrane (Zimmer et al. 2002). Thus, the reduction in Naᵥβ4 mRNA could contribute to the reduction in Na⁺ current.

**Functional significance of Na⁺ channels in vestibular hair cells**

**Spiking in immature hair cells.** In immature cochlear inner hair cells, spontaneous and evoked spiking is robust and repetitive (Marcotti et al. 2003). The spikes have a strong Ca²⁺ component; INa₂-like currents reduce the time to reach spike threshold, speed the rate of rise of spikes and increase spike frequency. Around the onset of hearing (P12), the spikes disappear as K⁺ channels are acquired and Na⁺ channels are lost. Comparable changes in rodent utricular hair cells, including the acquisition of gK,L, reduction in INa,1 and loss of INa,2, occur in concert with development of vestibular function; the vestibular-ocular reflex changes gradually during the first three postnatal weeks (Curthoys 1979, 1982). INa,2 may be considered a marker of immaturity in rodent hair cells.

In rat utricular hair cells, spikes occurred as single events evoked by a depolarization and usually requiring a pre-hyperpolarization. For cells with INa,2, the dependence of the spike on prepulse voltage was consistent with the INa,2 inactivation range. From the average inactivation curve in 1.3 mM Ca²⁺, 13% of INa,2 channels would be available for activation at our average resting potential of −59 mV. Similar single spikes were eliminated with TTX (Chabbert et al. 2003) or NMDG⁺ substitution for external Na⁺ (Géléoc et al. 2004), suggesting that Na⁺ currents play an essential role in spiking in rodent utricular hair cells, unlike the mouse inner hair cells (Marcotti et al. 2003). But differences in the recording conditions (temperature and Ca²⁺ buffering) may have affected the relative Ca²⁺ contributions.
The notion that spiking is an important part of neuronal development is widely believed. The Ca\(^{2+}\) component of spikes may activate hair cell transcription factors that promote survival, differentiation and synaptogenesis (Tao et al. 2002). It also directly promotes transmitter release (Marcotti et al. 2003), which may produce similar activity-dependent transcriptional changes postsynaptically. By boosting hair cell spikes, Na\(^{+}\) channels should enhance these effects. Chabbert et al. (2003) further proposed that Na\(^{+}\) channels influence inner ear synaptogenesis via neurotrophin release. In mouse vestibular organs, hair cell-afferent synapse formation depends on BDNF release from the epithelia (Ernfors et al. 1994). Application of TTX to immature rat utricular maculae substantially reduced electrically-evoked BDNF release, implying that voltage-gated Na\(^{+}\) channels boost activity-dependent BDNF release (Chabbert et al. 2003).

The puzzle of very negatively inactivating Na\(^{+}\) currents. At typical hair cell resting potentials of −50 to −70 mV, \(I_{\text{Na,1}}\) channels are almost fully inactivated, casting doubt upon their functionality. We suggest three possible answers to this puzzle. First, \textit{in vivo} resting potential may be more negative than measured experimentally. In olfactory neurons, which have Na\(^{+}\) currents with similarly negative inactivation ranges, Qu et al. (2000) argued that the resting potential must be unusually negative. Alternatively, the inactivation range may be more positive \textit{in vivo}. The Na\(^{+}\) channel inactivation range is susceptible to intracellular ATP (El Sherif et al. 2001; Choi et al. 2003), glycosylation (Tyrrell et al. 2001), \(\beta\) subunit expression, and temperature. In rat outer hair cells, \(V_{1/2,\text{inact}}\) values were −93 mV at room temperature and −85 mV at 36°C (Oliver et al. 1997). Such a shift could significantly increase the number of available Na\(^{+}\) channels at rest - unless resting potential shifts in parallel, as recorded in mouse utricular hair cells (Rüsch and Eatock 1996).

The third possibility is that although most channels are inactivated at resting potential the residual few still have an impact. This option is suggested by the diversity of conditions in which very negatively inactivating Na\(^{+}\) channels have been reported, including perforated-patch
recordings, semi-intact preparations, isolated hair cells, and animals for which room temperature is not aberrant (e.g. Evans and Fuchs 1987; Sugihara and Furukawa 1989). At −75 mV, as might occur during an after-hyperpolarizing potential (AHP) or during a negative deflection of the hair bundle, 8% of $I_{\text{Na},1}$ is activatable. But the time course of inactivation is shorter than the membrane time constant, such that a depolarizing response strong enough to activate $I_{\text{Na},1}$ would simultaneously inactivate it. This may explain why $I_{\text{Na},1}$ inactivation did not account for the changes in spiking with voltage in Figure 4.

The NMDG⁺-sensitive current of immature hair cells with $I_{\text{Na},1}$ and $I_{\text{Na},2}$ had a sustained component that may include persistent current, not previously noted in hair cells. Such currents prolong sub-threshold depolarizations (Prescott and De Koninck 2005), including those underlying complex spikes in cerebellar Purkinje cells (Raman et al. 1997) and pacemaking in subthalamic nucleus neurons (Do and Bean 2003). They might contribute to the repetitive firing of immature mouse inner hair cells (Marcotti et al. 2003), which might in turn produce intracellular $\text{Ca}^{2+}$ accumulation and $\text{Ca}^{2+}$ effects on differentiation.

**Variation with zone or hair cell type?** The variation in Na⁺ current in the immature rat utricular macula with zone and hair cell type may offer insights into its function. Zonal variation in transiently expressed ion channels could help set up the known physiological differences with zone in the mature epithelium. Central and peripheral zones of vestibular epithelia are clearly differentiated with respect to several aspects of synaptic morphology, from the numbers of complex calyces (e.g., Desai et al. 2005a,b) to the numbers and complexity of synaptic ribbons (Lysakowski and Goldberg 1997). Cell type differences raise the possibility that Na⁺ currents have a role in differentiating the afferent ending, which can form a simple calyx around one hair cell, a complex calyx around multiple hair cells, or a bouton ending.

The biggest zonal difference was the strong exclusion of $I_{\text{Na},2}$ from the striola during the first week. But terminal mitoses occur earlier in the central zones of vestibular epithelia (Sans and Chat 1982); might we have missed a prenatal peak in striolar $I_{\text{Na},2}$ expression? Data from Géléoc
et al. (2004) suggest not: in the ‘central zone’ of the embryonic mouse utricular macula, which should include striola, all Na⁺ currents were reported to be $I_{Na,1}$-like. Assuming that hair cells with $I_{Na,2}$ are more likely to spike than those with $I_{Na,1}$, the greater prevalence of $I_{Na,2}$ in the extrastriola and the results of Chabbert et al. (2003) suggest that there may be more hair-cell release of BDNF in the neonatal extrastriola.

All cells with calyces expressed $I_{Na,1}$, while our numbers are consistent with the possibility that all cells with $I_{Na,2}$ were immature type II cells. Is it possible that the underlying variation is by cell type and age, rather than zone, such that only cells destined to be type II cells ever express $I_{Na,2}$? In the mature rat utricle, hair cells in both zones are evenly divided with respect to type (Desai et al. 2005b). In the extrastriola in the first week, where all cells were unclassified because we could not detect calyces, the proportions (54% $I_{Na,2}$, 40% $I_{Na,1}$, 6% no current) are fairly consistent with a type I/ type II segregation. In the striola, however, 90% of all cells, and 79% of unclassified cells, had $I_{Na,1}$. This could only be reconciled with a strict type I/type II segregation of currents if we strongly selected for immature type I cells among the unclassified population. This is not out of the question, as their larger bundles and apical surfaces make them stand out.

In summary, we can be confident that type I cells express $I_{Na,1}$ channels at higher levels than $I_{Na,2}$ channels, at least from the onset of calyx formation, when we could first identify them as type I cells. If our sampling was unbiased with respect to type, our data suggest that in the striola, some immature type II cells preferentially express $I_{Na,1}$ channels, while in the extrastriola, most or all immature type II cells preferentially express $I_{Na,2}$ channels. Type II hair cells do show regional variation in K⁺ channel expression (Holt et al. 1999; Weng and Correia 1999; Masetto et al. 2000; Brichta et al. 2002).

**Na⁺ channels in afferents**
In vestibular ganglion neurons dissociated from P3-P6 mice, $\text{Na}^+$ currents resembled $I_{\text{Na},2}$ in TTX and voltage sensitivity (Chabbert et al. 1997). Such currents could be carried, at least in part, by $\text{NaV}_{1.2}$ and $\text{NaV}_{1.6}$ channels (Figs. 9B,C). In the mature mouse cochlea, Hossain et al. (2005) localized $\text{NaV}_{1.6}$ subunits to afferent endings below hair cells and nodes of Ranvier flanking the ganglion somata. Other TTX-sensitive subunits may contribute in rat vestibular afferents, given that all six TTX-sensitive subunits were expressed by P1 vestibular ganglia (Fig. 9).

Rennie and Streeter (2006) recorded $I_{\text{Na}}$ in calyx terminals dissociated from 3-12 week-old gerbils. Comparison of the biophysical and pharmacological properties of their current with those of the present study suggests that gerbil calyx endings have more than one $\text{Na}^+$ channel type: $V_{1/2,\text{inact}}$ values ($-83 \text{ mV}$) were halfway between the average values we obtained in hair cells for $I_{\text{Na},1}$ and $I_{\text{Na},2}$ (Fig. 2F); the slope factor (9.2 mV) was broad, which we have argued may reflect multiple channel types (Fig. 2E); and 100 nM TTX produced an 80% block, consistent with a mix of TTX-sensitive and –insensitive channels.

Our immunohistochemical results suggest that primary vestibular afferents express $\text{NaV}_{1.5}$ channels at calyx endings. In the heart, $\text{NaV}_{1.5}$ current helps set the frequency of pace-making (Lei et al. 2004). Certain afferents from the extrastriola and periphery of maculae exhibit ‘regular’ firing (reviewed in Goldberg 2000), a high-frequency pace-making. Regular firing could involve an AHP current (Smith and Goldberg 1986) and an $I_{\text{Na},1}$-like current relieved from inactivation by the AHP. $\text{NaV}_{1.6}$ or other subunits underlying persistent and resurgent current might also contribute to repetitive firing (Do and Bean 2003).

Immunostaining suggests that $\text{NaV}_{1.5}$ channels are present at high density in the calyx innerface membrane, where they would co-localize with $\text{K}^+$ channels of the KCNQ family (Kharkovets et al. 2000; Lysakowski and Price 2003; Hurley et al. 2006). This arrangement raises the possibility that afferent spikes originate in the post-synaptic membrane rather than further down the dendrite, as proposed at bouton endings (Hossain et al. 2005).
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Figure Legends

Figure 1. Rat utricular hair cells responded to depolarizing steps with transient currents carried by Na⁺. Steady-state inward currents were carried principally by Ca²⁺ but may have included a small Na⁺ component. Capacitance transients have been removed post-hoc. A-D, Whole-cell currents in an enzymatically isolated rat utricular hair cell with I_{Na,1} (P1); voltage protocol, bottom panel, voltage steps: –67, –47, –37, –7 mV. A. The cell was bathed locally with: A, Control: Cs⁺-SES, B, Cs⁺-SES with Na⁺ replaced by N-methyl-D-glucamine (NMDG⁺), and C, Wash: Cs⁺-SES. D, Difference current: (Wash – NMDG⁺). E, Peak current-voltage (I-V) relations from A-D (* fit with Boltzmann functions (Eq. 1, Materials and Methods) with the g_{max} term replaced by $\frac{I_{max}}{V - E_{Na}}$, where $I_{max}$ is the peak inward current and we use the calculated equilibrium potential for Na⁺ ($E_{Na}$) as an approximation of the current reversal potential. $V_{1/2}$, $S$ (mV): Control, –34.5, 9.3; Wash, –33.0, 8.8; Difference, –29.1, 10.3. The outward current in NMDG for strong depolarizations is presumably carried by Cs⁺ ions through incompletely-blocked K⁺ channels.

Figure 2. Two kinds of Na⁺ current with different voltage dependence of activation and inactivation. In this and subsequent figures, data are from the semi-intact preparation of the rat utricular macula unless otherwise specified. Holding potential, –67 mV. A, Activation: Inactivation was removed by a 50-ms prepulse to –137 mV, then Na⁺ currents were evoked by 20-ms depolarizing steps incremented in 5-mV steps. Thick trace, single exponential fit to the inactivation of the current elicited by –47 mV, $\tau = 1.1$ ms (see Fig. 3A). P2, striolar, unclassified hair cell type. B, Inactivation, same cell: 50-ms steps to various potentials were followed by a strongly activating test step to –17 mV. As prepulses became more positive, I_{Na} evoked by the
test step declined. **C.** Activation (filled symbols) and inactivation (open symbols) plots for two cells: the striolar one in A,B (circles) and a P3 lateral extrastriolar cell (triangles). Data were normalized to the peak current for each protocol and fit with the Boltzmann function (Eq. 1). Striolar cell: $V_{1/2,\text{inact}} S$ (mV): $-92.6, 5.0$; $V_{1/2,\text{act}} S$: $-43.4, 6.1$; Lateral extrastriolar cell: $V_{1/2,\text{inact}} S$: $-72.5, 5.1$; $V_{1/2,\text{act}} S$: $-32.3, 5.2$. A bimodal distribution of $V_{1/2,\text{inact}}$ values suggests two Na$^+$-current populations. Hair cells of P0-P4 utricular maculae. **D,G.** Histograms (1-mV bins) of $V_{1/2,\text{inact}}$ (D) and $V_{1/2,\text{act}}$ (G) values. **D.** The distribution of $V_{1/2,\text{inact}}$ values (106 cells) is fit with two Gaussians (Eq. 4, Materials and Methods) with midpoint ($V_c$) and width ($w$) values of $-92$ mV, $8.5$ mV and $-74$ mV, $4.7$ mV. We classified currents with $V_{1/2,\text{inact}}$ values negative to $-81$ mV (arrow) as $I_{\text{Na,1}}$ and other currents as $I_{\text{Na,2}}$. **E.** $S$ values from the Boltzmann fits, averaged across 3 1-mV bins. Numbers above data indicate number of cells. $S$ was $6.1 \pm 0.15$ mV (n = 46) for $V_{1/2,\text{inact}}$ values negative to $-90$ mV and $5.3 \pm 0.18$ mV (n = 26) for $V_{1/2,\text{inact}}$ values positive to $-75$ mV. In between (arrows), the mean and standard error of $S$ rose, peaking at $9.9 \pm 1.20$ mV (n = 6) at the trough in the $V_{1/2,\text{inact}}$ distribution (D). Cells with these large $S$ values and intermediate $V_{1/2,\text{inact}}$ values may have had appreciable amounts of each current. The inactivation curve (open circles) of one such cell (P4, lateral extrastriolar, unclassified hair cell type) is shown in **F,** together with a two-Boltzmann fit (thick line) using the $V_{1/2}$ and $S$ values for the mean inactivation curves for $I_{\text{Na,1}}$ and $I_{\text{Na,2}}$ (thin lines) and consistent with $47\%$ of the current being $I_{\text{Na,1}}$ and $53\%$ $I_{\text{Na,2}}$. $V_{1/2,\text{inact}}$ (mV), $S$ (mV), n cells for mean $I_{\text{Na,1}}$ curve: $-92 \pm 0.6$, $6.9 \pm 0.24$, 68; for mean $I_{\text{Na,2}}$ curve: $-74 \pm 0.5$, $6.0 \pm 0.24$, 38. $V_{1/2,\text{inact}}$, $S$ (mV) for the single-Boltzmann fit (not shown): $-80.3, 8.7$. **G.** Activation voltage ranges for $I_{\text{Na,1}}$ and $I_{\text{Na,2}}$ showed more overlap than the inactivation voltage ranges but also differed significantly. Gaussian fits of the $V_{1/2,\text{act}}$ distributions: $V_c$, $w$ (mV) - $I_{\text{Na,1}}$: $-38$, 8.8; $I_{\text{Na,2}}$: $-31$, 4.5.
Figure 3. The kinetics of $I_{Na,1}$ and $I_{Na,2}$ differed slightly. **A, B**, The voltage dependence of the mean inactivation time constants ($\tau_{inact}$, **A**) and time to peak ($t_{peak}$, **B**) for 6 cells for each current type. Logarithmic values for the two currents differed according to a two-way ANOVA (A, p<0.00001; B, p<0.001) and post-hoc Tukey means test (A, B, p<0.001). **C, D**, The time to recover from inactivation at the holding potential of −67 mV was determined by hyperpolarizing the cells to −137 mV for different durations (0 – 50 ms) before depolarizing to −17 mV. Scale bars: 250 pA, 10 ms (upper panel), 1 ms (lower panel). **C, INa,1; D, INa,2.** $I_{Na,2}$ was not fully inactivated at −67 mV (arrow). The time course of recovery of peak currents was fit with a single- or double-exponential function. Shown are ($\tau_1$, $\tau_2$, ms): $I_{Na,1}$, 0.91, 12; $I_{Na,2}$: 0.35, 23. The early phase of recovery is shown on an expanded time scale in the bottom traces.

Figure 4: $I_{Na,1}$ and $I_{Na,2}$ may play different roles in spiking. **A**, Voltage responses elicited by a 1-nA current step after a 200 ms prepulse between −100 and +180 pA (lower panel, **B**), from a P2 cell with $I_{Na,2}$ (peak Na$^+$ current in voltage clamp, −820 pA; input resistance 660 MΩ; zero-current potential −55 mV). The +1-nA step produced a larger depolarization when preceded by relatively hyperpolarized potentials (prepulse potentials; black traces) than with more depolarized prepulses (grey traces). Arrows in **A** and **B** point to shoulders in the black traces, suggestive of a threshold for spiking. **B**, Voltage responses elicited by the protocol in **A** from a P2 cell with $I_{Na,1}$ (peak Na$^+$ current in voltage clamp, −1.6 nA; input resistance 520 MΩ; zero-current potential −54 mV). **C,D**, Peak potential ($V_{peak}$) elicited by the +1-nA step plotted against prepulse potential (voltage averaged over a 2.5 ms-period preceding the +1-nA step) for the cells in **A,B** (filled circles) and the peak $I_{Na}$ inactivation data (as measured in Fig. 2) for the same cells (open triangles). **C,** The voltage range over which $V_{peak}$ was reduced overlapped with the inactivation range for the cell with $I_{Na,2}$. Boltzmann fits (lines): $V_{1/2}$, $S$ (mV) −62, 8.2 for $V_{peak}; −77, 11.2$ for
I_{Na,2} inactivation. **D,** There was no overlap between the inactivation range of I_{Na,1} in this cell and changes in $V_{peak}$. Boltzmann fit to inactivation data $V_{1/2} \sim -95$ mV, $S \sim 5.0$ mV.

**Figure 5.** $I_{Na,1}$ was TTX-insensitive; $I_{Na,2}$ was TTX-sensitive. Whole-cell recordings from enzymatically dissociated rat utricular hair cells (**A**, P2; **B**, P1). Current traces at $-55$ mV are bolded. Inactivation and activation ranges (**right**) were hyperpolarized relative to those recorded in the semi-intact preparation, possibly an effect of papain. **A,** 500 nM TTX blocked $I_{Na,1}$ by 56%. **B,** 50 nM TTX blocked $I_{Na,2}$ by 86%.

**Figure 6.** The two Na$^+$ currents were differently distributed in the major subdivisions of the utricular macula (P0 - P4). $V_{1/2, inact}$ data from Fig. 2D, replotted by region. **Right,** Surface view of the adult rat utricular macula, stained with calretinin antibody. **S,** striola; **MES** and **LES,** medial and lateral extrastriola. **A,** 90% of striolar cells but just 38% of extrastriolar cells had $I_{Na,1}$.  **B,** Almost all of the extrastriolar cells with $I_{Na,1}$ (16/18) were from the medial, rather than the lateral, extrastriola.

**Figure 7.** Changes in Na$^+$ current density (**A,B**) and incidence (**C,D**) with age. Current densities were calculated from the maxima of inactivation curves.  **A,B,** Na$^+$ current density by region and current type. Number of cells each day shown at top of histograms (**top row,** striola; **bottom row,** extrastriola). **A,** $I_{Na,1}$: **Striola:** Current density was approximately uniform from P0 to P9 and decreased by the third postnatal week. **Extrastriola:** Current density declined after P1, plateauing by P6 at 20% of the peak value. **B,** $I_{Na,2}$: **Extrastriola:** Mean current density in cells with $I_{Na,2}$ peaked at P3 and dropped 72% by P4. **Striola:** $I_{Na,2}$ was seen in just 6/87 striolar hair cells, but trends in current density with age resembled those in the extrastriola. 5/87 striolar and 13/97 extrastriolar hair cells (including 4 at P3-P4, not shown) lacked Na$^+$ current altogether.  **C,D,**
Incidences of $I_{Na,1}$, $I_{Na,2}$ and no $I_{Na}$ as functions of age. Striola: Most striolar hair cells expressed $I_{Na,1}$ across all ages, but from P13-P21, 28% (5/18) striolar cells had no current. The incidence of $I_{Na,2}$ in the striola was 11% (6/53) from P0-P6 and 0% (0/34) thereafter. D, The incidence of $I_{Na,2}$ in the extrastriola peaked at 65% (22/34) from P3-P4 and fell to 0% (0/20) from P14-P22.

**Figure 8.** Developmental changes in $I_{Na,1}$ current density, separated by cell type and zone ($A,B$) and inactivation range ($C$). A, Current density in type I cells, classified by the presence of a calyx or partial calyx. Numbers of cells given next to each point. B, Cells without calyces are considered unclassified for P<9 and as type II cells for P≥9. All cells in the extrastriola were unclassified at P<9. Unclassified ($B$) and type I ($A$) cells in the striola showed similar trends with age. C, $V_{1/2,inact}$ values became more negative at rates of 0.9 mV/day for $I_{Na,1}$ (filled circles, $r^2 = 0.44$) and 0.8 mV/day for $I_{Na,2}$ (open triangles, $r^2 = 0.25$). Thin lines, 95% confidence intervals.

**Figure 9.** Vestibular epithelia expressed mRNA for multiple Na$^+$ channel $\alpha$ and $\beta$ subunits at P1 and P21. Agarose gels of PCR products (40 amplification cycles: see Materials and Methods). $A,B$: Examples of PCR bands on agarose gels for each of 9 $\alpha$ subunits ($A,B$) and 4 $\beta$ subunits ($B$). Expected product sizes are given in Table 1. A, TTX-sensitive $\alpha$ subunits. Negative controls: $H_2O$, water control. –RT controls were done for each tissue (not shown). $\lambda$, 100 bp ladder (brightest band represents 500 bp). Test tissues: $U$, utricular epithelium; $C$, crista. Positive control for primers: $B$, brain; $H$, heart; $Sk$, skeletal muscle. Vestibular ganglia ($G$) were used as positive controls for Na$\gamma$1.7, Na$\gamma$1.8 and Na$\gamma$1.9 (see Materials and Methods). Positive controls for tissue: all tissues yielded strong bands for the calcium channel subunit, Ca$\gamma$1.3 (not shown). PCR products were detected for Na$\gamma$1.1, Na$\gamma$1.2 and Na$\gamma$1.6 in utricular epithelium and crista at P1 and P21. Products for Na$\gamma$1.3 were only detected in crista at P21. Products for Na$\gamma$1.4 and Na$\gamma$1.7 were detected in vestibular epithelia at P1 but not at P21. $B$, TTX-insensitive subunits...
and β subunits. PCR products were obtained for Na\textsubscript{v}1.5 for both epithelia at both ages; products for Na\textsubscript{v}1.8 and Na\textsubscript{v}1.9 were not found in vestibular epithelia at both ages. PCR products for most β subunits were obtained from utricular macula and crista at both P1 and P21, with the exception of Na\textsubscript{v}β4 in the utricular macula at P21 and Na\textsubscript{v}β2 in the crista at P21. The primer set for Na\textsubscript{v}β4 also recognized otogelin (upper band in P1 crista, confirmed by sequencing), a glycoprotein expressed in the extracellular matrices of the inner ear (Cohen-Salmon et al. 1997).

C, Histograms showing the incidence of PCR products in multiple experiments. Top, bottom rows of numbers above bars: numbers of preparations (experiments) at P1 and P21, respectively.

D, PCR bands on 10% acrylamide gel for three splice variants (right) of Na\textsubscript{v}1.6 in P1 rat: adult (18A), neonatal (18N) and a variant with exon 18 missing (Δ18). The functional adult form is only seen in vestibular ganglia. E, Some single hair cells from P1 utricular macula showed expression of Na\textsubscript{v}1.5 and Na\textsubscript{v}1.2 subunits. 7.5% acrylamide gels of PCR products (40 amplification cycles: see Materials and Methods). PCR products for β-actin (lower panel) were obtained from each cell as a positive control for the quality of the collection. Positive controls for primers: P1 ganglion (G), Na\textsubscript{v}1.2; P1 utricular macula (U), Na\textsubscript{v}1.5. Numbers above and below gels represent individual hair cells. Striolar cells: 1, 2, 5, 6, 7, 8, 11; extrastriolar cells: 3, 4, 9, 10. Two striolar (1, 8) and one extrastriolar (4) cells were positive for Na\textsubscript{v}1.5. Three striolar (1, 6, 8) and one extrastriolar (4) cells were positive for Na\textsubscript{v}1.2. Note the expression of two Na\textsuperscript{+} channel α subunits in 3 of the 4 cells.

Figure 10. Na\textsubscript{v}1.5-, Na\textsubscript{v}1.2- and Na\textsubscript{v}1.6-like immunoreactivity in hair cells and afferent fibers in the utricular macula. Confocal sections taken at ages indicated in the lower left corner of each panel. Controls (top panels in each column): Pre-absorption with 10-fold excess of the peptide used to generate the antiserum eliminated staining. Scale bar, top left panel, 10 μm, applies to all panels. The striola (s) is located approximately between the short vertical lines.
Left, Na\textsubscript{v}1.5.  

\textit{P1 - P3:} Hair cell cytoplasm was diffusely stained; examples shown with \textit{single arrowheads}, or, if brightly stained, \textit{double arrowheads}. 

\textit{Asterisks} (P1, P3) show the unstained supporting cells at the level of their nuclei. 

Some afferent fibers below the epithelium also stained (\textit{thick short arrows}). 

\textit{P6-P21:} Brightly stained hair cells were no longer seen; light hair cell staining persisted (\textit{single arrowheads}). 

Brightest staining was in primary vestibular afferents, at multiple sites: the calyx inner face, with especially bright staining in narrow bands at the interface of two hair cells within a complex calyx (P6, \textit{thin arrow}); afferents below the epithelium (P21, \textit{thick short arrows}), including the calyx-only afferents (identified by their calretinin staining, not shown); and the afferents’ cell bodies.

Middle, Na\textsubscript{v}1.2.  

\textit{P0-P8:} Hair cell cytoplasm stained diffusely (\textit{arrowheads} point to examples). 

Supporting cells show less staining at the nuclear level (\textit{asterisks}, P0, P3) but bright staining at the apical surface of the epithelium (\textit{open arrows, all panels}). 

Labeling of hair bundles at P8 and P21 is likely spurious; hair bundles are known to be sticky in immunocytochemical preparations.

\textit{P21:} Labeling was very light.

Right, Na\textsubscript{v}1.6.  

\textit{P0-P8:} Hair cell cytoplasm stained diffusely (\textit{single white arrowheads} point to examples). 

The brightest staining was at P3 (\textit{double arrowheads}), except for patches of bright stain in the necks of hair cells (\textit{black on white arrowheads}; see also Fig. 11D). 

\textit{P21:} Hair cells were largely unstained. Apical surfaces of supporting cells stained (\textit{open arrow}).

\textbf{Figure 11.} Na\textsubscript{v}1.5-, Na\textsubscript{v}1.2- and Na\textsubscript{v}1.6-like immunoreactivity in hair cells and afferent fibers in the utricular macula at high resolution. 

\textit{Left panels:} Na\textsuperscript{+} channel antisera alone; \textit{Right panels:} Na\textsuperscript{+} channel antisera plus calretinin antibody (\textit{Cal, green}). 

In the first few postnatal days, calretinin antibody labels many, but not all, hair cells and no supporting cells. 

\textit{Scale bar} (\textit{A, left}) 10 \textmu m applies to all panels. 

\textit{A, Na\textsubscript{v}1.5, striola, P1:} most hair cells labeled with Na\textsubscript{v}1.5 antibody (\textit{red}), but at different intensities. 

A brightly stained nascent calyx (\textit{thin arrow}) envelopes a brightly stained type I hair cell (\textit{double arrowhead}). 

Afferent dendrites also stain brightly (\textit{thick arrowheads}). 

61
arrows). Thin arrow points to bright staining between hair cells in a complex calyx. Single arrowhead points to a hair cell with less intense staining. Supporting cells did not label (asterisk). B, Na\textsubscript{v}1.5, striola, P21. Hair staining for Na\textsubscript{v}1.5 decreased (single arrowhead) but afferent staining was strong (thick arrow) C, Na\textsubscript{v}1.2, striola, P3: Most hair cells labelled well (double arrowheads). Supporting cells were not stained at the nuclear level, but some were strongly stained at their apices, which have triangular profiles (open arrow). D, Na\textsubscript{v}1.6, striola, P0: Both hair cells and supporting cells were stained above the supporting cell nuclear level (asterisks). In many hair cells (black and white arrowheads), there were bright clumps of label between the cuticular plate and the nucleus.

Figure 12. Na\textsubscript{v}1.5-like immunoreactivity in the whole mount P21 utricular macula. A, striola; B, extrastriola. Scale bar (A), 10 \textmu m applies to both panels. Single confocal transverse sections (large square panels) at the level of hair cell nuclei are flanked by orthogonal slices (top and side rectangles) at the levels indicated (green and red lines), reconstructed from z-stacks (A, 23-\textmu m stack; B, 17.5-\textmu m stack) with Zeiss LSM510 software. Blue lines in side rectangles show the z-level within the stack (corresponding to the level shown in the square panel). Identifying symbols (arrowheads, etc.) in the square panel are repeated in the orthogonal slices, so that each type of symbol points to the same structure viewed from different angles. In the striola (A), the green calretinin antibody labeled calyces belonging to calyx-only afferents and some type II cells; in the extrastriola (B) calretinin antibody labeled many type II cells. Na\textsubscript{v}1.5 antibody (red) stained calyces (rings around type I hair cells in the square sections) and some type I hair cells (I) and type II hair cells (II). The ring-like structures reflect label in the calyx inner faces, and possibly in hair cell membranes. Asterisks, hair cells with no Na\textsubscript{v}1.5-like immunoreactivity. In the striola (A), arrowheads point to yellow label, reflecting co-localization of calretinin-like immunoreactivity and Na\textsubscript{v}1.5-like immunoreactivity in calyces. Na\textsubscript{v}1.5 label was strongest at interfaces between hair cells in complex calyces (arrows in A). In orthogonal slices in B, arrows
show that Na\textsubscript{v}1.5-like immunoreactivity in calyces was co-extensive with labeling of afferent stalk below the hair cell. There was also punctate staining within hair cells, including some type II cells (II) where it often overlapped with calretinin to produce yellow dots (A,B, \textit{black on white arrowheads}).

\textbf{Table 1.} PCR Primers. Primers were either designed or modified from others previously published (Na\textsubscript{v}1.1(a), Na\textsubscript{v}1.6(a): Schaller and Caldwell 2000; Na\textsubscript{v}1.1(b), Na\textsubscript{v}1.3: Alessandri-Haber et al. 2002; Na\textsubscript{v}1.6(b): Plummer et al. 1997; Na\textsubscript{v}1.8: Dib-Hajj et al. 1998a; Na\textsubscript{v}1.9: Dib-Hajj et al. 1998b; β-actin: Raff et al. 1997; single-cell primers: Na-deg (degenerate primer) and Na\textsubscript{v}1.2: Chabbert et al. 2003).
Table 1: PCR Primers

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<td>NaV1.5</td>
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<td>5'-AGATGTTGACCTCTCTGAG-3'</td>
<td>256</td>
</tr>
<tr>
<td><strong>CaV1.3 (control primer set)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaV1.3</td>
<td>5'-TGAGACACAGACAGACAGAAGC-3'</td>
<td>5'-GGTGTACCTGGGTGATCTG-3'</td>
<td>345</td>
</tr>
</tbody>
</table>
A

Na,1.1  Na,1.2  Na,1.3  Na,1.4  Na,1.6  Na,1.7

P1

P21

H,O  λ  U  C  B  U  C  B  U  C  B  Sk  U  C  B  U  C  G

B

Na,1.5  Na,1.8  Na,1.9  Na,β1  Na,β2  Na,β3  Na,β4

P1

P21

H,O  λ  U  C  H  U  C  G  U  C  G  U  C  B  U  C  B  U  C  B

C

Utricle

Crista

D

E

Na,1.5  Na,1.2  Na,1.2

β-actin

18A  18N  Δ18

H,O  U  λ  λ  U  λ  λ  U  λ  λ  U  λ  λ  U  λ  λ  U  λ  λ