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26. Iron was provided as ferric chloride ($FeCl_3$) and added at final concentrations of 10 nM, 100 nM, 1 μM, and 10 μM. All the polycarbonate containers used to make ASW and to store the stock solutions were precleaned with 10% HCL-Milli Q water (1-week treatment) and subsequently washed with Milli Q water. All solutions were prepared in Milli Q water. Iron limitation was achieved by transfer of an exponentially growing culture (5 ml) initially containing 10 μM $FeCl_3$ (normal ASW concentrations) into 100 ml of ASW medium containing 0.1 mM EDTA with different iron concentrations (10 nM, 100 nM, 1 μM, and 10 μM $FeCl_3$). Experiments were performed only after a late-log-phase culture had passed through at least five successive rounds of growth for 7 days each under different iron-starvation media conditions. All experiments were repeated five times. The amount of dissolved iron (0.2 μm filtered) was determined with graphite furnace atomic absorption spectrophotometry. Measurements of Fe′ were made by means of adsorptive cathodic stripping voltammetry with 2-(2-thiazolylazo)-p-cresol [P. A. M. Farias *et al.*, *Anal. Lett.* **25**, 1929 (1992)].
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Cholinergic Synaptic Inhibition of Inner Hair Cells in the Neonatal Mammalian Cochlea

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Efferent feedback onto sensory organs provides a means to modulate input to the central nervous system. In the developing mammalian cochlea, inner hair cells are transiently innervated by efferent fibers, even before sensory function begins. Here, we show that neonatal inner hair cells are inhibited by cholinergic synaptic input before the onset of hearing. The synaptic currents, as well as the inner hair cell's response to acetylcholine, are mediated by a nicotinic (α9-containing) receptor and result in the activation of small-conductance calcium-dependent potassium channels.

In the mature mammalian cochlea, inner hair cells (IHCs) transduce acoustic signals into receptor potentials and communicate to the brain by synaptic contact with as many as 20 unbranched afferent fibers (*1*). In contrast, outer hair cells (OHCs) have few afferent contacts but are the principal target of cholinergic olivocochlear efferents. However, before the onset of hearing [about postnatal day 11 in rats (*2*)], a transient efferent innervation is found on IHCs, even before olivocochlear fibers contact the OHCs. These transient contacts have been documented in several species (*3*), suggesting that at least some of this innervation might be cholinergic; however, these synapses have never been shown to be functional nor have IHCs been shown to be sensitive to acetylcholine (ACh).

We used whole-cell recording of IHCs in acutely excised apical turns of the rat organ of Corti (postnatal days 7 to 13) (*4*) to examine the functional role of the transient synaptic contacts on immature IHCs. Because efferent axons are cut in this preparation, we relied on spontaneous transmitter release. Spontaneous transient currents were observed in 57 rat IHCs (~50% of IHCs tested) (Fig. 1). These occurred at rates of 0.5 per minute up to 15 per second and persisted for up to 10 min. At −80 mV, the spontaneous currents were inward with an amplitude of 16 ± 4 pA, rose rapidly (time to peak, 5 ± 2 ms), and fell more slowly (decay time constant, 20 ± 5 ms; 214 events analyzed in eight IHCs) (Fig. 1A). At −30 mV, somewhat larger and longer lasting outward currents were observed with an amplitude of 35 ± 9 pA, a time to peak of 19 ± 3 ms, and a decay time constant of 30 ± 4 ms (122 events analyzed in four IHCs). At intermediate voltages, the waveform was biphasic with an inward peak followed by a longer lasting outward current (Fig. 1B). The estimated reversal of the later outward current was around −80 mV, suggesting that this component was carried by potassium (equilibrium potential E_K at −82 mV).

We elevated extracellular potassium from 5.8 to 15 mM to depolarize synaptic terminals (Fig. 2A) (*5*). This caused a steady inward current in IHCs voltage-clamped to −80 mV (as expected from the resting potassium conductance with E_K now at −58 mV). In addition, both the frequency and amplitude of the spontaneous currents were increased by this change. The increased frequency is presumably due to increased transmitter release from depolarized efferent terminals. The increase in amplitude arose in part from the larger potassium driving force at −80 mV, but also possibly was affected by increased multiquantal release as well.

Given the probable synaptic origin of the spontaneous currents, we next examined their pharmacology. The biphasic waveform of the currents is reminiscent of ACh-evoked currents in hair cells of chicks and mammals (*6*). There, an early inward cation current flows through α9-containing ACh receptors. The Ca^{2+} influx through these channels then activates Ca^{2+}-dependent small-conductance (SK) potassium channels and thereby induces an outward current. Thus, we tested the ability of α-bungarotoxin (α-BTX) and strychnine, antagonists of the α9 receptor, to affect the spontaneous currents. Spontaneous inward currents were completely blocked by 300 nM α-BTX at −90 mV within 25 to 40 s (n = 3) (Fig. 2B). Although we could not demonstrate washout for α-BTX, the time course of the block was similar to that for the block of exogenously applied ACh (Fig. 3). Within 1 s, 1 μM strychnine completely blocked spontaneous currents (n = 4). Within 20 s, 100 nM strychnine blocked spontaneous currents, and currents were recovered after washout (n = 2) (Fig. 2C); in one experiment, block and recovery was induced twice. The SK channel blocker apamin reduced spontaneous currents in four IHCs within 15 and 45 s. Outward spontaneous currents at −30 mV were eliminated by 1 or 10 nM apamin in two IHCs (Fig. 2D). Inward currents at −90 mV were reduced in amplitude by 1 nM apamin in two IHCs. The residual current at −90 mV is presumed to flow through the ACh receptors, which are themselves cation channels and insensitive to apamin.

We next determined how IHCs respond to ACh. At −80 mV, 100 μM ACh caused inward currents in 53 of 55 IHCs with amplitudes between 20 and 400 pA (Fig. 3A).

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Despite the limitation imposed by the slow perfusion of the intact cochlear coil, in one cell at −60 mV the ACh response was biphasic (Fig. 3B), revealing an early inward current before the larger outward current. The ACh-evoked current reversed in sign at −67 ± 8 mV (n = 7). The current-voltage (I-V) relation was bell shaped, with the current amplitude diminishing at 0 mV (Fig. 3C). With 10 mM 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) in the whole-cell pipette solution [instead of ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA)], the I-V relation lost its bell shape and reversed positive to 0 mV.

These results are consistent with the hypothesis that ACh activates a combination of ACh-gated cation current and Ca^{2+}-activated potassium current in IHCs. This was also supported by pharmacological tests (Fig. 3D). ACh-evoked currents in IHCs were blocked reversibly by 300 nM α-BTX (at −30 mV, n = 2; at −80 mV, n = 3) or by 100 nM strychnine (at −30 mV, n = 2). At −30 mV, 100 pM apamin eliminated the ACh-evoked outward current (n = 3). At −90 mV, 1 μM apamin reduced the total current by >50% (n = 3), with the remainder due to cationic flux through the ACh receptor.

Early postnatal IHCs generate slowly repetitive calcium action potentials (Fig. 4A) (*7*). In addition, we observed occasional hyperpolarizing potentials that correspond to the spontaneous synaptic currents seen in voltage clamp. These inhibitory synaptic potentials seemed to delay or prevent spontaneous action potentials, but this was difficult to quantify. We thus examined the effect of exogenously applied ACh on IHC activity. The frequency of ongoing action potentials was reduced by 10 μM ACh (Fig. 4B), and 20 μM ACh completely eliminated IHC action potentials and produced a 15-mV hyperpolarization (Fig. 4C). Comparing these effects of ACh to spontaneous hyperpolarizations (Fig. 4A) suggests that synchronous evoked synaptic release from efferent neurons could strongly modulate the firing frequency of IHCs and afferent neurons with which they synapse.

Immature neurons in the auditory nerve and central nuclei fire low-frequency bursts of action potentials spontaneously (*8*) or in response to sound (*9, 10*). The firing becomes continuous when the efferent input is cut in neonatal cats (*9*), implying that the cholinergic inhibition of IHCs imposes rhythmicity onto the immature auditory pathway. Coordinate bursting of afferent fibers could influence activity-dependent synaptic differentiation, as suggested for retinogeniculate connections in the visual pathway (*11*). The efferent modulation of IHCs may be unique to the neonatal cochlea. After the onset of hearing, the spontaneous action potentials in IHCs disappear, and at postnatal day 21, we found no IHC response to ACh (n = 5).

We have shown that immature IHCs within the rat organ of Corti are subject to synaptic inhibition through a biphasic ionic mechanism like that established by the application of ACh to isolated hair cells (*6*). Similar biphasic synaptic effects have been observed during efferent inhibition of hair cells in the turtle (*12*), suggesting that this cholinergic mechanism may be common among vertebrate hair cells. The pharmacology of hair cell inhibition is also well conserved and consistent with that of the nicotinic receptor α9 expressed in oocytes (*13*). The mRNA of α9 is expressed in the IHCs of rats, guinea pigs, and mice (*13–15*), and hair cells in the chick express the avian ortholog (*16*). Although homomeric α9 can form functional channels in oocytes, it remains to be determined whether the native synaptic response is similarly constituted or if it requires additional subunits. IHCs do not express mRNA for α2 through α7 (*15*), but recently, a related nicotinic subunit, α10, has been identified in rats (*17*) and humans (*18*). To address these issues using transgenic mice, we have replicated the experiments described here in IHCs of BALB/c mice and found that biphasic synaptic currents and

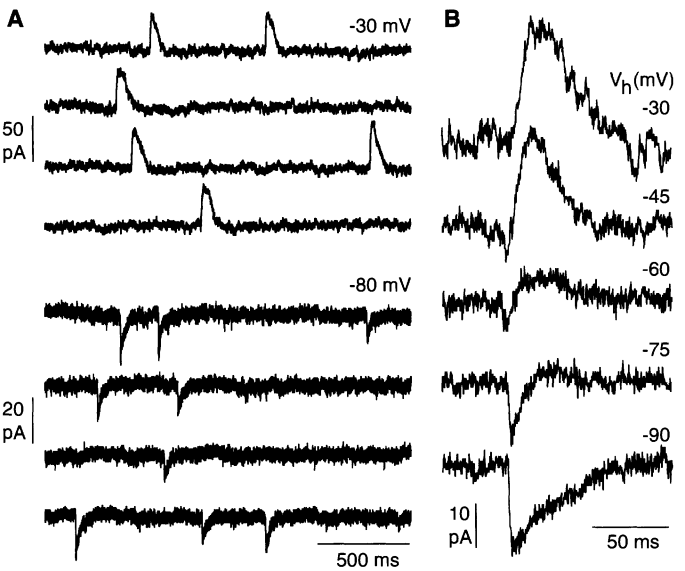

Fig. 1. Spontaneous synaptic activity in postnatal rat IHCs. Whole-cell currents at different holding potentials (V_h). (**A**) Outward currents at −30 mV and inward currents at −80 mV recorded in a 12-day-old IHC. (**B**) Single events recorded at different holding potentials in a 10-day-old IHC. Waveforms are biphasic at −75, −60, and −45 mV.

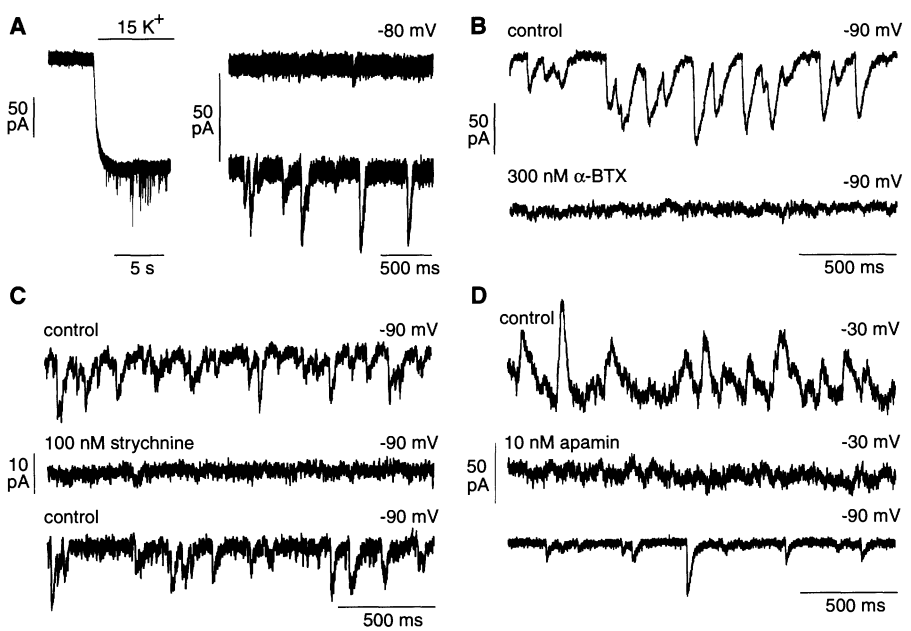

Fig. 2. Elevation and block of synaptic currents in IHCs. (**A**) At −80 mV, extracellular potassium was raised from 5.8 to 15 mM, inducing a steady inward current of 140 pA and enhancing amplitude and frequency of the synaptic currents (see magnified traces). (**B**) Inward currents (in 15 mM extracellular potassium) were blocked by 300 nM α-BTX. (**C**) Inward currents were reversibly blocked by 100 nM strychnine. (**D**) At −30 mV, outward currents were blocked by 10 nM apamin. At −90 mV, inward currents persisted in apamin.

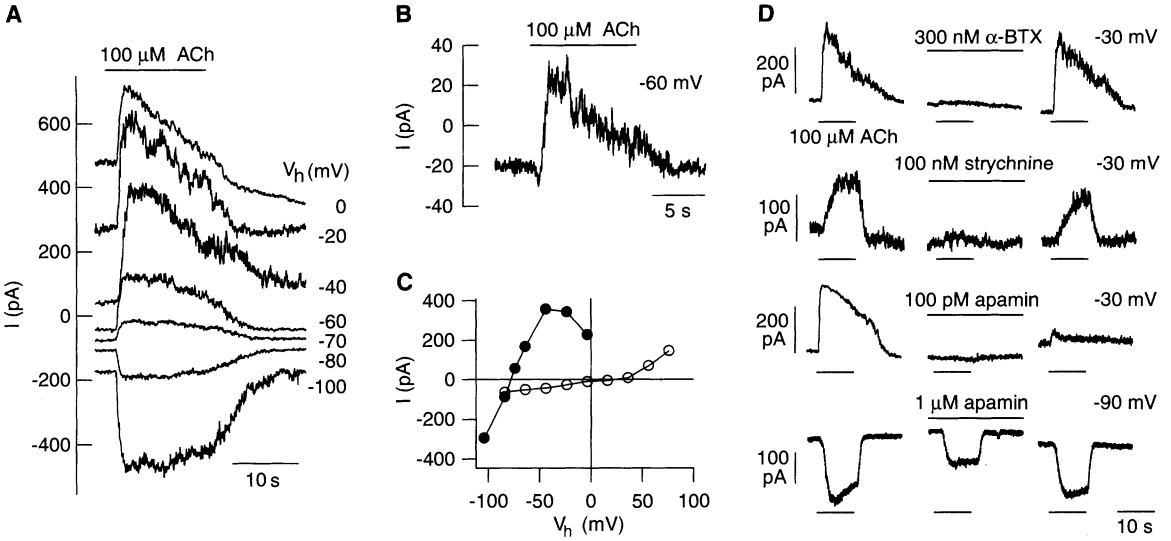

Fig. 3. ACh-activated currents in IHCs. Every 2 min, 100 μM ACh was applied for 10 to 20 s. (**A**) ACh response at different holding potentials. (**B**) Biphasic response at −60 mV showed that a small inward current is followed by a larger outward current. (**C**) *I-V* curves of two cells with different intracellular Ca^{2+} buffers (solid circles, 5 mM EGTA; open circles, 10 mM BAPTA). (**D**) Blocking the ACh response. At −30 mV, 300 nM α-BTX, 100 nM strychnine, and 100 pM apamin reversibly blocked ACh-activated currents. At −90 mV, 1 μM apamin blocked 56% of the total current.

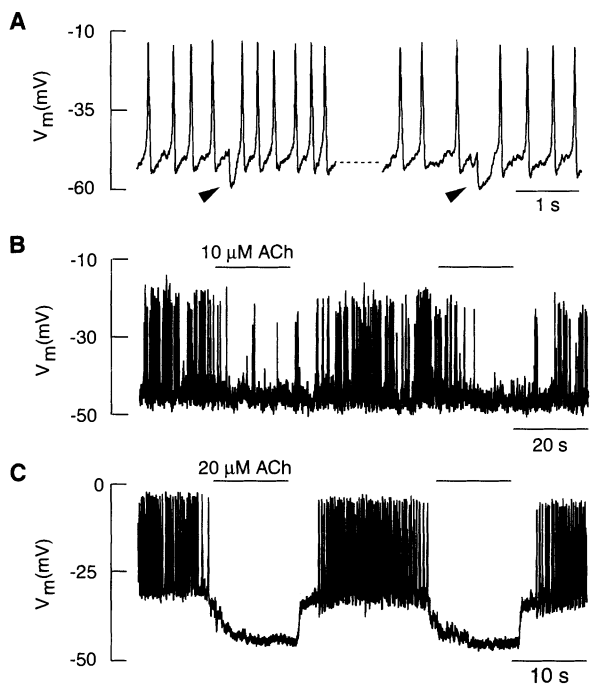

Fig. 4. Cholinergic inhibition of IHC action potentials. To elicit frequent firing of Ca^{2+} action potentials, we constantly injected current (I_{inj}) into IHCs. (**A**) Spontaneously occurring currents (arrows) hyperpolarized the membrane potential (V_m) by ~11 mV and thereby delayed the generation of Ca^{2+} action potentials; I_{inj} = 120 pA. (**B**) With 10 μM ACh, the firing rate of Ca^{2+} action potentials was reduced by a 1-mV hyperpolarization from a membrane potential of −45 mV; I_{inj} = 100 pA. (**C**) With 20 μM ACh, the membrane potential was hyperpolarized from −30 to −45 mV, thereby abolishing the generation of Ca^{2+} action potentials; I_{inj} = 120 pA.

cholinergic sensitivity are essentially identical to those seen in rats (*19*). The synaptic inhibition of cochlear hair cells through α9-containing ACh receptors provides an essential experimental model for understanding the function of this class of nicotinic cholinergic receptors.

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4. Apical turns of the organ of Corti were excised from Sprague-Dawley rats and used within 3 hours. IHCs were identified in the turn with a ×63 water immersion objective and during recordings by the size of their capacitance (7 to 11 pF), by their characteristic Ca^{2+} action potentials and voltage-dependent Na^+ and K^+ currents, and at older stages including a fast-activating K^+ conductance (*7*). Some cells were removed to access IHCs, but mostly the pipette moved through tissue under positive pressure. Extracellular solution (in mM) was as follows: 155 NaCl, 5.8 KCl, 0.9 $MgCl_2$, 1.3 $CaCl_2$, 0.7 NaH_2PO_4, 5.6 D-glucose, and 10 Hepes; pH 7.4. Pipette solution (in mM) was as follows: 150 KCl, 3.5 $MgCl_2$, 0.1 $CaCl_2$, 5 EGTA, 5 Hepes, and 2.5 Na_2ATP (ATP, adenosine 5′-triphosphate); pH 7.2. Resistances of the pipettes were 5 to 10 megohms. The indicated holding potentials were corrected for liquid junction potentials (−4 mV) for the *I-V* relations only (Fig. 3C). Voltages were not corrected for the voltage drop across the uncompensated series resistance. Experiments were done at room temperature (20° to 25°C). Data were filtered at 2 kHz and digitized at 5 or 20 kHz with PClamp6 software.
5. Extracellular solutions with elevated potassium or diluted drugs were applied with a glass pipette (150-μm tip diameter) positioned at ~300 μm from the recorded IHC. Application of elevated potassium changed membrane potentials with time constants of ~1 s.
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