Supporting Information

Lipovsek et al. 10.1073/pnas.1115488109

SI Materials and Methods

All experimental protocols were carried out in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals, as well as guidelines of the Institutional Animal Care and Use Committees of Johns Hopkins University and Instituto de Investigaciones en Ingeniería Genética y Biología Molecular.

Expression of Recombinant Receptors in *Xenopus laevis* Oocytes. Rat α9 and α10 cDNAs constructed in pSGEM, a modified pGEMHE vector suitable for *Xenopus laevis* oocyte expression studies, were used as described previously (1). Chicken α9 was derived from a pcDNA3.1 clone (2) and subcloned in pSGEM. Chicken α10 cDNA was PCR-amplified from a chicken basilar papilla cDNA library built in the Uni-ZAP XR vector (Stratagene). The lambda phage library was heated at 100 °C for 5 min. The primers used (sense, GGATCCACTAGTGACGGCCCCACTGCAATG; antisense, GAATTCATCGATGTCAGGCAGCTTTGCCAATC) were designed to hybridize with the 5′ and 3′ UTRs of the chicken α10 cDNA according to GenBank sequence NM_001101036. PCR analyses were done in a reaction mixture composed of 0.25 U of PCR enzyme mix (Fail Safe; Epicentre), 10 μL of Failsafe Premix D, and 5 μM of each primer in a MJ Research PTC-200 thermal cycler (Bio-Rad). Cycle parameters were 95 °C for 5 min, followed by 35 cycles of 1 min at 95 °C, 1 min at 67 °C, and 2 min at 72 °C; and then a final cycle of 10 min at 72 °C. PCR reactions were checked on an agarose gel, purified with a QIAquick PCR purification kit (Qiagen), sequenced for verification of correct amplification, and sublconed in the pSGEM vector. Rat α4 and β2 subunit cDNAs subcloned into pBS SK(−) (Stratagene) were kindly provided by Dr. Jim Boulter (University of California, Los Angeles, CA).

Capped cRNA was transcribed in vitro using the Promega RiboMAX Large-Scale RNA Production System, with plasmid linearized with NheI for rat and chicken α9 and α10, EcoRI for α4, and SacI for β2.

X. laevis frogs were obtained from Nasco. The maintenance of stage V and VI oocytes has been described in detail elsewhere (3–5). In brief, oocytes were surgically removed from the ovaries of *X. laevis* frogs anesthetized with 3-aminobenzoic acid ethylester (~1 g/mL), then incubated with 2 mg/mL of Worthington type I collagenase for 4 h in Ca^{2+}-free saline solution [96 mM NaCl, 2 mM KCl, 5 mM $MgCl_2$, 5 mM Hepes (pH 7.6)], with slow agitation to remove the follicular cell layer. Oocytes were then washed extensively in the same solution and maintained at 18 °C in Barth's solution comprising 88 mM NaCl, 1 mM KCl, 0.91 mM $CaCl_2$, 24 mM $NaHCO_3$, 0.33 mM $Ca(NO_3)_2$, mM 0.82 $MgSO_4$, and 10 mM Hepes (pH 7.6), supplemented with 100 mg/mL of gentamicin sulfate. Typically, oocytes were injected with 50 nL of RNase-free water containing 0.01–1.0 ng of cRNAs (at a 1:1 molar ratio) and maintained in Barth's solution at 18 °C.

Electrophysiological recordings were performed at 2–6 d after cRNA injection under a two-electrode voltage-clamp with a Geneclamp 500 amplifier (Molecular Devices). Both voltage and current electrodes were filled with 3 M KCl and had a resistance of ~1–2 MΩ. The preparation was grounded by means of an Ag/AgCl wire in a 3 M KCl solution connected to the bath through a 3 M KCl agar bridge. During electrophysiological recordings, oocytes were continuously superfused (~10 mL/min) with normal frog saline composed of 115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, and 10 mM Hepes buffer (pH 7.2). Drugs were applied in the perfusion solution of the oocyte chamber. The solution used for the divalent cation permeability studies was 10 mM Hepes, 0.2–5 mM Ca^{2+}, and 100–120 mM *N*-methyl-*D*-glucamine to compensate for changes in osmolarity, with pH adjusted to 7.2 with HCl. In all of the experiments, oocytes were superfused for 2 min with the test solution before the application of ACh and were transferred back to normal frog saline (1.8 mM Ca^{2+}) for at least 3 min before changing to a different test solution.

To minimize the activation of the native oocyte's Ca^{2+}-sensitive chloride current, $I\mathrm{Cl}_{\mathrm{Ca}}$ (6–8), by Ca^{2+} entering through nAChRs, unless stated otherwise, all experiments were carried out in oocytes incubated with the membrane permeant Ca^{2+} chelator 1,2-bis (2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid-acetoxymethyl ester (BAPTA-AM; 100 μM) for 3 h before electrophysiological recording was performed. This treatment was previously shown to effectively chelate intracellular Ca^{2+} ions and thereby impair activation of the $I\mathrm{Cl}_{\mathrm{Ca}}$ (9). To minimize activation of the oocyte's nonselective inward current through a hemigap junction channel in response to the reduction of the external divalent cation concentration, all experiments were carried out in oocytes injected with 7.5 ng of an oligonucleotide antisense (5′-GCTTTAGTAATTCCCATCCTGCCATGTTTC-3′) to connexin C38 mRNA (10, 11).

Current-voltage (I-V) relationships were obtained by applying 2-s voltage ramps from −120 to +50 mV at the plateau response to 10 or 100 μM ACh from a holding potential (V_{hold}) of −70 mV. Leakage correction was performed by digital subtraction of the I-V curve obtained by the same voltage ramp protocol before the application of ACh. Voltage protocols and data acquisition were generated using a Digidata 1200 digitizer and pClamp 7.0 software (Molecular Devices). Data were analyzed using Clamp Fit from pClamp 7.0 and Graph Pad Prism 4.00 for Windows (GraphPad Software).

Concentration-response curves were normalized to the maximal agonist response in each oocyte. Response is reported as percentage of the response to ACh. The mean ± SEM of peak current responses are represented. Agonist concentration-response curves were iteratively fitted with the equation $I/I_{max} = A^n/(A^n + EC_{50}{}^n)$, where I is the peak inward current evoked by agonist at concentration A, I_{max} is the current evoked by the concentration of agonist eliciting a maximal response, EC_{50} is the concentration of agonist inducing half-maximal current response, and n is the Hill coefficient.

Chicken Basilar Papilla. The auditory organ (basilar papilla) was dissected from the temporal bone of embryonic chickens (white Leghorns, 17–20 d in ovo). After 5–10 min of exposure to protease (Sigma-Aldrich Type IV, 0.1 mg/mL) in buffered saline, the tegmentum vasculosum and tectorial membrane were removed, exposing the sensory hair cells. The basilar papilla was secured to a coverslip by small spring clips (made from fine insect pins) and transferred to a recording chamber on the stage of a Zeiss Axioskop 2 and viewed with differential interference contrast using a 40× water immersion objective and a camera with contrast enhancement (Hamamatsu C2400-07). Short hair cells were recorded in a region corresponding to ~500–1,000 Hz along the tonotopically organized basilar papilla of the chicken (0.25–0.5 mm, the distance from the apical tip toward the base). These cells were confirmed to be short hair cells by their position farthest from the neural limbus, where they receive predominantly efferent innervation (12). Furthermore, the voltage-dependent membrane conductance was dominated by short hair cell-specific "A type" rapidly inactivating potassium current (13). Finally, these cells responded to ACh with a combination of ligand-gated cation

current followed by Ca^{2+}-dependent potassium current through apamin-sensitive ("SK-like") potassium channels, as demonstrated previously (14). Patch and puffer pipettes [3–5 MΩ, coated with Sylgard silicon elastomer (Dow Corning) and fire-polished] were mounted on piezoelectric manipulators (Burleigh PCS 5000) for positioning on hair cells. Voltage-clamp recordings were obtained using an Axopatch 200B running under Clampex software (Molecular Devices). Subsequent data analysis was carried out with Clampfit, Excel, and Origin software. Recordings were made at room temperature. External and pipette solutions were as used in previous experiments in rat inner hair cells (15). I-V curves were constructed by changing the hair cell membrane potential from −100 mV to +100 mV in 2-s steps of 10 mV, or by a 200-ms voltage ramp over that same range. ACh (1 mM) was pressure-ejected (Picospritzer; General Valve Corporation) for 200–500 ms from a nearby "puffer" pipette (~1-μm opening), resulting in ACh-evoked currents of variable waveform, but usually lasting 0.5–1 s. Control I-V values were subtracted from those obtained in the presence of ACh to measure the reversal potential of the ACh-evoked current.

To measure reversal potentials as a function of external calcium, we used an extracellular solution containing 100 mM NaCl, 5.8 mM KCl, 0.1–10 mM $CaCl_2$, 5.6 mM d-glucose, and 10 mM Hepes buffer (pH 7.4), with osmolality adjusted to 300–320 mosmol kg^{-1} with sucrose. To measure current through the cationic nAChR only, without contaminating SK current, we used a pipette solution containing 140 mM CsCl, 3.5 mM $MgCl_2$, 10 mM BAPTA-AM, 5 mM Hepes buffer, and 2.5 mM Na_2ATP, with pH adjusted to 7.2 with CsOH. Apamin (300 nM) was added to the external saline to further minimize calcium-activated SK currents. In these experiments, also designed to study the voltage dependence of the cationic nAChR currents and/or the changes in the current reversal potential, KCl was replaced by CsCl to eliminate voltage-dependent K^+ currents (16).

SK Current Measurement in Chicken and Rat Hair Cells. To measure ACh-evoked SK potassium currents, we obtained patch-clamp recordings from inner hair cells in apical turns of the cochlea excised from young (P9–P10) rats, in which efferent synaptic input is commonly found (17, 18), and from chicken hair cells obtained as above. Rat and chicken hair cell recordings were obtained under identical ionic conditions. The external (bath) solution contained 144 mM NaCl, 5.8 mM KCl, 0.9 mM $MgCl_2$, 1.3 mM $CaCl_2$, 0.7 mM NaH_2PO_4, 10 mM Hepes, and 5.6 mM glucose, with pH adjusted to 7.4 with NaOH. The internal (pipette) solution contained 135 mM KCl, 3.5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 5 mM EGTA, 5 mM Hepes, 2.5 mM Na_2ATP, with pH adjusted to 7.2 with KOH (~150 mM total K^+). ACh (100 μM) in external saline was pressure-applied (300 ms) from a nearby (20–30 μm) puffer pipette (tip diameter ~1 μm). Steady-state current voltage curves were obtained from both cell types, showing that the amplitude of ACh-evoked potassium current varied with membrane potential, reversing near the potassium equilibrium potential. At membrane potentials positive to +20 mV, ACh fails to evoke potassium current, consistent with a requirement for calcium influx. The experimental protocol exploited that feature by initiating ACh-evoked potassium current at the permissive voltage of −40 mV, then stepping to +40 mV near the peak of the response to greatly reduce calcium influx, causing a decline of the potassium current. The voltage protocol was then repeated in the absence of ACh and the voltage-evoked membrane current subtracted from the recording obtained during ACh exposure. The ACh-evoked potassium current declined approximately exponentially at +40 mV in all hair cells; thus, for the purpose of comparison, time constants were obtained by best fit of a single exponential to the first (dominant) component of current decay.

Calcium Permeability. The relative Ca^{2+} to monovalent permeability (pCa/pMono) was evaluated as described previously for recombinant and native mammalian α9α10 receptors (3, 5, 15, 19), by analyzing the shift in the reversal potential (E_{rev}) as a function of the increase in the extracellular Ca^{2+} concentration. In the case of oocytes, taking into account that the ICl_{Ca}, although minimized by the treatment with BAPTA-AM, could increase with increasing external Ca^{2+} concentration and thus reduce the rightward shift in the apparent E_{rev} (E_{Cl}, ~ −25 mV), we replaced all monovalent cations in the extracellular medium by the impermeant monovalent cation *N*-methyl-*D*-glucamine to enhance the shift in E_{rev} on variations in Ca^{2+}, as described previously (3, 5, 19). The internal concentrations of Na^+ and K^+ used in the calculations for oocytes were 20 mM and 150 mM, respectively. Permeability ratios were calculated for each oocyte and then averaged. This was not possible for chicken hair cells, because these cells did not last enough to be tested at all different Ca^{2+} concentrations. For chicken hair cells, we assumed that Na^+, K^+, and Cs^+ were equally permeant and that Cl^- was impermeant through this channel; in addition, we made no allowance for the effect of surface charge on the value of pCa/pMono (20). We calculated the relative pCa/pMono by the Goldman-Hodgkin-Katz (GHK) constant field voltage equation assuming no anion permeability and extended to include divalent cations (21, 22), as reported previously for rat and human receptors (3, 5, 15, 19).

Molecular Evolution Analysis. All sequences used, including those of the *CHRNA4*, *CHRNB2*, *CHRNA7*, and *CHRNA10* genes of 23 vertebrate species, were downloaded from GenBank (www.ncbi.nlm.nih.gov/genbank) and Ensembl (www.ensembl.org) databases. Alignments of sequences used for the evolutionary analysis are shown in Fig. S5, and accession numbers are listed in Table S2. All sequences were visually inspected, and missing and/or incorrect exons were obtained from the NCBI Genome Project *traces* database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence alignment for each of the four genes was performed using the ClustalW tool implemented in Vector NTI 10 software (www.invitrogen.com) and manually edited using MEGA4 software (www.megasoftware.net).

To test for the presence of positive selection, we applied the branch-site test of positive selection developed by Yang and coworkers (23–25) using the codeml program implemented in PAML. The branch-site model allows ω, the ratio of nonsynonymous to synonymous substitution rate, to vary both among sites in the protein and across branches on the tree and requires the a priori specification of foreground (the branch of interest) and background (all other branches) lineages within the phylogeny. Accordingly, the analysis assigns each site in the protein to one of three site classes. Class 0 and 1 sites include codons that are conserved ($0 < \omega_0 < 1$) or neutral ($\omega_1 = 1$) throughout the tree and have the same ω value in the background and foreground lineages; codons assigned to class 2 sites are conserved or neutral on the background branches but become under positive selection on the foreground branches, with $\omega_2 > 1$. The test aims to detect positive selection affecting a few sites along the foreground lineage (the class 2 sites) by comparing the branch-site model (H_A), in which class 2 sites are under positive selection (with $\omega_2 > 1$), to a null model (H_0) in which class 2 sites are neutral (with $\omega_1 = 1$) through a likelihood ratio test (LRT). The LRT (twice the log likelihood difference between the two models) is compared with a χ^2 distribution with the degrees of freedom equal to the difference in parameters between the two models.

Phylogenetic Reconstruction. Phylogenetic trees were built using MEGA4 (26). Amino acid sequences were downloaded from GenBank and Ensembl databases and aligned using ClustalW software (27). All positions containing alignment gaps and missing data were eliminated only in pairwise sequence com-

parisons. The final dataset contained a total of 764 positions. The evolutionary distances (i.e., number of amino acid substitutions per site) were computed using the JTT matrix-based method (28). The neighbor-joining algorithm (29) was used to generate the initial tree, after which the evolutionary history was inferred using the minimum evolution method (30).

Statistics and Reagents. Statistical significance was evaluated using the Student *t* test (two-tailed, unpaired samples). A P value < 0.05 was considered significant. ACh chloride (purchased from Sigma-Aldrich) was dissolved in distilled water as a 100 mM stock and stored as aliquots at −20 °C. BAPTA-AM (Sigma-Aldrich) was stored at −20 °C as aliquots of a 100 mM solution in dimethyl sulfoxide, thawed, and diluted 1,000-fold in Barth's solution shortly before incubation of the oocytes. Apamine and salts where also purchased from Sigma-Aldrich. The SERCA inhibitor tert-benzo-hydroquinone (Tocris) was dissolved in dimethyl sulfoxide for perfusion into the bath. Control solutions contained equivalent levels of dimethyl sulfoxide.

1. Elgoyhen AB, et al. (2001) alpha10: Aa determinant of nicotinic cholinergic receptor function in mammalian vestibular and cochlear mechanosensory hair cells. *Proc Natl Acad Sci USA* 98:3501–3506.
2. Hiel H, Luebke AE, Fuchs PA (2000) Cloning and expression of the alpha9 nicotinic acetylcholine receptor subunit in cochlear hair cells of the chick. *Brain Res* 858:215–225.
3. Katz E, et al. (2000) High calcium permeability and calcium block of the α9 nicotinic acetylcholine receptor. *Hear Res* 141:117–128.
4. Elgoyhen AB, Johnson DS, Boulter J, Vetter DE, Heinemann S (1994) Alpha 9: An acetylcholine receptor with novel pharmacological properties expressed in rat cochlear hair cells. *Cell* 79:705–715.
5. Weisstaub N, Vetter DE, Elgoyhen AB, Katz E (2002) The alpha9alpha110 nicotinic acetylcholine receptor is permeable to and is modulated by divalent cations. *Hear Res* 167:122–135.
6. Barish ME (1983) A transient calcium-dependent chloride current in the immature *Xenopus* oocyte. *J Physiol* 342:309–325.
7. Boton R, Dascal N, Gillo B, Lass Y (1989) Two calcium-activated chloride conductances in *Xenopus laevis* oocytes permeabilized with the ionophore A23187. *J Physiol* 408:511–534.
8. Miledi R, Parker I (1984) Chloride current induced by injection of calcium into *Xenopus* oocytes. *J Physiol* 357:173–183.
9. Gerzanich V, Anand R, Lindstrom J (1994) Homomers of alpha 8 and alpha 7 subunits of nicotinic receptors exhibit similar channel but contrasting binding site properties. *Mol Pharmacol* 45:212–220.
10. Arellano RO, Woodward RM, Miledi R (1995) A monovalent cationic conductance that is blocked by extracellular divalent cations in *Xenopus* oocytes. *J Physiol* 484:593–604.
11. Ebihara L (1996) *Xenopus* connexin38 forms hemi-gap-junctional channels in the nonjunctional plasma membrane of *Xenopus* oocytes. *Biophys J* 71:742–748.
12. Zidanic M (2002) Cholinergic innervation of the chick basilar papilla. *J Comp Neurol* 445:159–175.
13. Murrow BW, Fuchs PA (1990) Preferential expression of transient potassium current (IA) by "short" hair cells of the chick's cochlea. *Proc Biol Sci* 242:189–195.
14. Fuchs PA, Murrow BW (1992) Cholinergic inhibition of short (outer) hair cells of the chick's cochlea. *J Neurosci* 12:800–809.
15. Gómez-Casati ME, Fuchs PA, Elgoyhen AB, Katz E (2005) Biophysical and pharmacological characterization of nicotinic cholinergic receptors in rat cochlear inner hair cells. *J Physiol* 566:103–118.
16. Kros CJ, Ruppersberg JP, Rüsch A (1998) Expression of a potassium current in inner hair cells during development of hearing in mice. *Nature* 394:281–284.
17. Glowatzki E, Fuchs PA (2000) Cholinergic synaptic inhibition of inner hair cells in the neonatal mammalian cochlea. *Science* 288:2366–2368.
18. Katz E, et al. (2004) Developmental regulation of nicotinic synapses on cochlear inner hair cells. *J Neurosci* 24:7814–7820.
19. Sgard F, et al. (2002) A novel human nicotinic receptor subunit, α10, that confers functionality to the α9 subunit. *Mol Pharmacol* 61:150–159.
20. Mayer ML, Westbrook GL (1987) Permeation and block of N-methyl-D-aspartic acid receptor channels by divalent cations in mouse cultured central neurones. *J Physiol* 394:501–527.
21. Lewis CA (1979) Ion-concentration dependence of the reversal potential and the single channel conductance of ion channels at the frog neuromuscular junction. *J Physiol* 286:417–445.
22. Jan LY, Jan YN (1976) L-glutamate as an excitatory transmitter at the *Drosophila* larval neuromuscular junction. *J Physiol* 262:215–236.
23. Yang Z (2007) PAML 4: Phylogenetic analysis by maximum likelihood. *Mol Biol Evol* 24:1586–1591.
24. Yang Z, Nielsen R (2002) Codon-substitution models for detecting molecular adaptation at individual sites along specific lineages. *Mol Biol Evol* 19:908–917.
25. Zhang J, Nielsen R, Yang Z (2005) Evaluation of an improved branch-site likelihood method for detecting positive selection at the molecular level. *Mol Biol Evol* 22: 2472–2479.
26. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software, version 4.0. *Mol Biol Evol* 24:1596–1599.
27. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22: 4673–4680.
28. Jones DT, Taylor WR, Thornton JM (1992) The rapid generation of mutation data matrices from protein sequences. *Comput Appl Biosci* 8:275–282.
29. Saitou N, Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4:406–425.
30. Rzhetsky A, Nei M (1992) A simple method for estimating and testing minimum evolution trees. *Mol Biol Evol* 9:945–967.

Table S1. Fit of Ca^{2+} permeability data for recombinant receptors

	rn α9α10	gg α9α10	rn α4β2
Linear fit			
Slope	39.3 ± 0.4	13.2 ± 5.2	9.7 ± 9.4
Different from 0	Yes (P = 0.0001)	No (P = 0.856)	No (P = 0.374)
GHK model fit			
pCa/pMono	10.3 ± 0.9	ND	ND
R^2	0.769	−4.788	−5.135
Shift in E_{rev} from Ca 0.2 to 2 mM	40 ± 8.6	3.6 ± 11.3	−7.5 ± 13.8
Number of experiments	5	8	5

ND, not determined because of lack of fit to the GHK equation. Note that for rat α9α10 receptors, a 10-fold increase in $Ca2^+$ concentration from 0.2 to 2 mM resulted in a 40 ± 8.6 (n = 5) mV shift in E_{rev}. Moreover, the fit of the data to a straight line had a slope of 39.3 ± 0.4 (n = 5), which was significantly different from 0 (P = 0.0001), illustrating the high degree of dependency of E_{rev} with extracellular $Ca2^+$ concentration. In contrast, the shifts in reversal potential were negligible in the case of α4β2 receptors. A 10-fold increase in $Ca2^+$ concentration from 0.2 to 2 mM resulted in a −7.5 ± 13.8 mV (n = 5) shift in E_{rev}, which was not significantly different from 0, and the fit of the data to a straight line gave a slope of 9.7 ± 9.4 (n = 5), which again did not differ from 0 (P = 0.374).

Table S2. Accession numbers for sequences used in the molecular evolution analyses

Species	*CHRNA4*	*CHRNB2*	*CHRNA7*	*CHRNA10*
Human (*Homo sapiens*)	NM_000744	NM_000748.2	NM_000746.3	NM_020402.2
Chimp (*Pan troglodytes*)	NM_001034114	XM_001152392.1	ENSPTRP00000011728	ENSPTRT00000006124
Marmoset (*Callithrix jacchus*)	ENSCJAT00000019780	XM_002760042.1	*	ENSCJAT00000030096
Rat (*Rattus norvegicus*)	NM_024354	NM_019297.1	NM_012832.3	NM_022639.1
Mouse (*Mus musculus*)	NM_015730	NM_009602.4	NM_007390.3	NM_001081424
Cow (*Bos taurus*)	XM_617408	NM_001192474.1	NM_174515.2	XM_585021
Dolphin (*Tursiops truncates*)	ENSTTRT00000001826	ENSTTRT00000013999	ENSTTRT00000002864	ENSTTRT00000016638†
Microbat (*Myotis lucifugus*)	*	*	Not included	ENSMLUT00000013585†
Megabat (*Pteropus vampyrus*)	ENSPVAT00000014108†	*	ENSPVAT00000017796†	ENSPVAT00000016436
Cat (*Felis catus*)	*N*-SCAN chrA3.4.036.a	*N*-SCAN chrF1.9.069.a	ENSFCAP00000002072†	*
Dog (*Canis familiaris*)	XM_543097	XM_547565.2	XM_545813.2	XM_542333.2
Elephant (*Loxodonta africana*)	ENSLAFT00000005403	ENSLAFT00000017396	ENSLAFP00000007506	ENSLAFT00000013049†
Armadillo (*Dasypus novemcintus*)	Not included	*	ENSDNOT00000011829†	ENSDNOT00000002691†
Kangaroo (*Macropus eugenii*)	ENSMEUT00000006748†	ENSMEUT00000003737	Not included	ENSMEUT00000009591†
Opossum (*Monodelphis domestica*)	ENSMODT00000021412	XM_001373116.1	XM_001377478	ENSMODT00000010443
Platypus (*Ornitorhyncus anatinus*)	ENSOANT00000005905†	ENSOANT00000014865†	NW_001687589†	*
Lizard (*Anolis carolinensis*)	ENSACAT00000005848	ENSACAT00000015666	*	ENSACAT00000002286
Chick (*Gallus gallus*)	NM_204814	NM_204813.1	NM_204181.2	AJ295624
Finch (*Taeniopygia guttata*)	ENSTGUT00000007753	ENSTGUT00000004261	*	Not included
Frog (*Xenopus tropicalis*)	NM_001113843.1	NM_001100214.1	ENSXETT00000035796	ENSXETT00000010653†
Zebrafish (*Danio rerio*)	NM_001048063	XM_685308.4	ENSDART00000051931	ENSDART00000012872
			ENSDART00000077953	ENSDART00000012037
Medaka (*Oryzias latipes*)	ENSORLT00000001059	ENSORLT00000012169	ENSORLT00000023459	ENSORLT00000004999
			ENSORLT00000023451	ENSORLT00000016545†
Fugu (*Takifugu rubripes*)	AY299462.1	AY297428.1	AY298751.1	AY299471
			AY298752.1	AY299472
			AY298753.1	

*Sequence assembled through a Blast search at the NCBI Genome Project traces database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).
†Sequence corrected and/or completed using trace fragments obtained from the NCBI Genome Project traces database.

Fig. S1. Chicken α9 and α10 subunits assemble into functional receptors. (*A*) Concentration-response curves to ACh for rat and chicken α9α10 nAChRs. Responses were normalized to the maximal response in each oocyte. Values are mean ± SEM (n = 6 per point). (*Inset*) Representative responses to 3, 10, and 100 μM ACh obtained in an oocyte injected with chicken α9 and α10 cRNAs. (Scale bars: vertical, 10 nA; horizontal, 25 s). (*B*) Representative traces illustrating desensitization of responses to a 1-min application of 100 μM ACh for rat and chicken α9α10 nAChRs. After a peak response, inward currents decayed in the presence of ACh. At a holding potential of −70 mV, the current remaining at 20 s after the peak response in the presence of ACh was similar for rat (64.4% ± 3.6% of peak value; n = 5) and chicken (63.6% ± 3.7% of peak value; n = 19) α9α10 nAChRs.

Fig. S1

Fig. S2. I-V curves for ACh-evoked currents in chicken hair cells. (*A*) Representative traces of responses to a puff (500 ms) application of 1 mM ACh at different holding potentials. (*B*) I-V plots. Gray squares represent membrane control currents; gray triangles, ACh-evoked currents; black triangles, cholinergic currents resulting from the difference between control and ACh-evoked currents. (*C*) Representative I-V curves obtained on application of a 200-ms voltage ramp. Control membrane current (light-gray line) was subtracted from the I-V curve obtained in the presence of ACh (gray line) to obtain the cholinergic I-V curve (black line). Recordings are representative of eight cells in which ramp and step protocols were compared directly.

Fig. S2

Fig. S3. Phylogenetic tree of vertebrate nAChR subunits. Coding sequences for the *CHRNA1-10*, *CHRNB1-4*, *CHRND*, *CHRNG*, and *CHRNE* genes from *Homo sapiens* (human), *Rattus novergicus* (rat), *Mus musculus* (mouse), *Bos taurus* (cow), *Canis familiaris* (dog), *Gallus gallus* (chick), *Xenopus tropicalis* (frog), *Danio rerio* (zebrafish), and *Takifugu rubripes* (fugu) were used to reconstruct the phylogenetic relationships of vertebrate nAChR subunits. The optimal tree with a sum of branch length of 22.13853833 is shown. For the sake of clarity, the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown only next to the branches that separate different subunits. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the tree. Note that mammalian α10 subunits form an outgroup of the α9/α10 branch. In contrast, in all other subunits, the mammalian group is located within the corresponding subunit subbranch.

Fig. S3

Fig. S4. Alignment of transmembrane (TM) regions 1 and 2 of α10 nAChR subunits. The thick gray bars at the top indicate TM1 and TM2. The TM2 α-helix lines the pore of a nAChR channel. Numbering of TM2 residues follows the scheme proposed by Miller (1). Residues −1′, 16′, and 17′, which are involved in the Ca^{2+} permeability of α7, α4β2, and/or α3β4 receptors, are shaded in gray.

Fig. S4

Fig. S5. Sequences and alignments used for evolutionary analysis of the α4, β2, α7, and α10 subunits. Alignments were performed separately for each subunit using ClustalW and manually edited in MEGA4. The figure was generated with GeneDoc software (http://www.nrbsc.org/gfx/genedoc). Amino acids are shaded according to a conservation mode that emphasizes the degree of conservation in each column in the alignment: black, dark gray, light gray, and white shadings indicate 100%, >80%, >60%, and <60% conservation, respectively. The signal peptides are not included in the sequences. Each subunit comprises a large N-terminal extracellular domain that includes the superfamily characteristic Cys loop and the pair of double cysteines that define the α subunits (denoted by a star). The four TM domains are represented by gray bars.

Fig. S5