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A Point Mutation in the Hair Cell Nicotinic Cholinergic Receptor Prolongs Cochlear Inhibition and Enhances Noise Protection

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The transduction of sound in the auditory periphery, the cochlea, is inhibited by efferent cholinergic neurons projecting from the brainstem and synapsing directly on mechanosensory hair cells. One fundamental question in auditory neuroscience is what role(s) this feedback plays in our ability to hear. In the present study, we have engineered a genetically modified mouse model in which the magnitude and duration of efferent cholinergic effects are increased, and we assess the consequences of this manipulation on cochlear function. We generated the Chrna9L9 T line of knockin mice with a threonine for leucine change (L9 T) at position 9 of the second transmembrane domain of the α9 nicotinic cholinergic subunit, rendering α9-containing receptors that were hypersensitive to acetylcholine and had slower desensitization kinetics. The Chrna9L9 T allele produced a 3-fold prolongation of efferent synaptic currents in vitro. In vivo, Chrna9L9 T mice had baseline elevation of cochlear thresholds and efferent-mediated inhibition of cochlear responses was dramatically enhanced and lengthened: both effects were reversed by strychnine blockade of the α9x10 hair cell nicotinic receptor. Importantly, relative to their wild-type littermates, Chrna9L9 /T mice showed less permanent hearing loss following exposure to intense noise. Thus, a point mutation designed to alter α9x10 receptor gating has provided an animal model in which not only is efferent inhibition more powerful, but also one in which sound-induced hearing loss can be restrained, indicating the ability of efferent feedback to ameliorate sound trauma.


Introduction

In bringing information about the world to an individual, sensory systems perform a series of common functions. Each system responds with some specificity to a stimulus, and each one employs some specialized receptor cells at the periphery to translate specific stimuli into electrical signals that all neurons can use. That initial electrical event begins the process by which the central nervous system constructs an orderly representation of, for example, sounds, odors, tastes, and objects. Thus, basic sound detection begins when sound waves strike the ear drum, which transmits that physical stimulus to the organ of Corti within the cochlea, the sensory epithelium of the mammalian inner ear. Here, the primary receptor cells known as inner hair cells (IHCs) transform the information into electrical signals that are sent to the central nervous system by the auditory nerve [1]. However, unlike vision, touch, and the chemical senses, sound processing is modulated by efferent signals that travel in reverse, from the brain back to the inner ear [2]. One fundamental question in auditory neuroscience is what role(s) this feedback plays in our ability to hear.

The medial olivocochlear (MOC) efferents (Figure 1A) originate in the medial portion of the superior olivary complex and project to outer hair cells (OHCs; Figure 1B) of the organ of Corti, where large synaptic contacts are formed [2]. In contrast to IHCs, OHCs act as biological motors to amplify the sound-evoked motions of the organ of Corti through a type of somatic electromotility generated by a specialized membrane protein known as prestin. Activation of the MOC pathway, either by sound or by shock trains delivered to the bundle at the floor of the IVth ventricle (Figure 1A), reduces cochlear sensitivity through the action of the neurotransmitter acetylcholine (ACh) on nicotinic re-
Author Summary

Nicotinic cholinergic receptors are essential to higher order brain function. Structurally, these operate through a myriad of ligand-gated pentameric arrangements of different homologous subunits. Here, we report progress in understanding the structural properties of a neuronal nicotinic receptor at the synapse. Receptors assembled from two nicotinic cholinergic subunits (α9 and α10) serve exclusively at the synapse between central nervous system descending fibers and cochlear hair cells. This enabled us to show direct causality between a point mutation of the α9 subunit, and predicted alterations in the synaptic strength in sensory hair cells of the cochlea of α9 point mutant mice. Furthermore, this single mutation results in profound enhancement of central nervous system feedback to the cochlea. And finally, as a consequence, mutant mice possessing this altered receptor have substantially improved resistance to traumatic sound. Thus, central neuronal feedback on cochlear hair cells provides an opportunity to define one specific role that nicotinic receptors can play in the nervous system, enabling study from biophysical to behavioral levels and promoting a target for the prevention of noise-induced hearing loss.

In addition to their significance in auditory processing, efferent cholinergic synapses provide a unique opportunity to assess the function of neuronal nAChRs. In contrast to nAChR-expressing cells in the central nervous system, hair cells are compact and isopotential, and receive no other synaptic inputs. Moreover, compared to most other neuronal nAChRs, whose subunit composition remains challenging to elucidate [11], the oligomeric structure of the hair cell nAChR has been defined: α9 and α10 subunits arrange into a pentameric assembly with a likely (α9)2(α10)3 stoichiometry [12–15]. Current data support the notion that activation of the α9α10 nAChR leads to an increase in intracellular Ca2+ and the subsequent opening of small conductance Ca2+-activated K+ SK2 channels, thus leading to hyperpolarization of hair cells [3,16,17]. (Figure 1C).

In the present study, we have generated a genetically modified mouse model in which the magnitude and duration of the MOC efferent effect is increased, and assessed the consequences of this manipulation on auditory thresholds and susceptibility to noise-induced hearing loss. We have substituted a threonine for a leucine at position 9’ (L9’T) of the second transmembrane domain of the α9 subunit and examined the neurotransmitter responses, synaptic efficiency, and cochlear function of Chrna9Cebtf, Chrna9G4L9 T, and Chnra9G4L9 T mice. We show that the L9’T mutation produced an increase in sensitivity to ACh, and decreased rates of ACh-induced desensitization. More significantly, synaptic currents were dramatically prolonged in the OHCs of Chnra9G4L9 T mice. Consistent with these effects, Chnra9G4L9 T mice had elevated acoustic thresholds, and shock-evoked MOC activation produced both enhanced and prolonged cochlear suppression. This enhanced efferent inhibitory drive attenuated sound-induced, permanent acoustic injury. Our results establish efferent feedback inhibition’s function in preventing acoustic trauma, and provide preliminary characterization of a mouse model to explore additional physiological functions of MOC innervation. In addition, it motivates further exploration of the efferent synapse as a model system to study fast neurotransmission mediated by a neuronal nAChR.

Results

The targeting construct and generation of Chrna9L9’T mutant mice are described in the Materials and Methods section and Figure 2. Both Chrna9G4L9 T and Chnra9G4L9 T mutant mice were viable through adulthood, fertile, showed the expected gender and Mendelian genotype ratios, and exhibited no overt behavioral phenotype. Gross morphology of the cochlear duct was normal as seen in light microscopic evaluation of plastic sections of osmium-stained cochleae.
As assessed by quantitative real-time PCR (RT-PCR), the expression levels of cochlear nAChR α9 subunit mRNA were similar in Chrna9<sup>wt/wt</sup> and Chrna9<sup>L9T/L9T</sup> mice (Table S1). In addition, the expression levels of genes encoding proteins known to localize to the olivocochlear (OC) synaptic complex (for example, the nAChR α10 subunit and SK2 channel) were not changed in mutant mice (Table S1). Lastly, the electrophysiology of IHCs and OHCs was normal, as indicated by similar voltage-dependent K⁺ currents (Figure S2).

Hypersensitive and Slowly Desensitizing nAChRs in Mutant Mice

In Xenopus oocytes, α9α10 nAChRs assembled from mutant α9L9T subunits exhibit an increased sensitivity to ACh, a reduced desensitization rate, and an increased channel mean open time [18]. To determine whether similar changes occurred in hair cells of L9T mutant mice, we examined the response of neonatal IHCs to exogenous ACh. IHC nAChRs, which are expressed transiently before the onset of hearing, are of the α9α10 subtype and are functionally indistinguishable from those that arise later in OHCs [19,20].
Since recordings are more frequently and reliably obtained from neonatal IHCs than OHCs, the former provided the bulk of the data presented. As shown below, a subset of our findings has been repeated in OHCs of Chrna9L9/T mutant mice.

As observed in Figure 3A, IHCs of Chrna9pto/pto and Chrna9L9/T mice responded to ACh at P6–P10. In each case, ACh dose-response curves of IHCs were left-shifted when compared to wild type, with a concomitant decrease in the half maximal effective concentration (EC50). The EC50 was significantly different (p < 0.01) between homozygous mutant and wild-type IHCs (Chrna9pto: EC50, 90.8 ± 18.2 μM, Hill coefficient 1.6 ± 0.1, n = 4 animals, 7 cells; Chrna9L9/T: EC50, 57.2 ± 6.5 μM, Hill coefficient 2.2 ± 0.1, n = 4 animals, 9 cells; and Chrna9L9/T: EC50, 41.7 ± 5.7 μM, Hill coefficient 2.1 ± 0.2, n = 4 animals, 7 cells). During prolonged (>60 s) application of 1 mM ACh at −90 mV (Figure 3B), the current was entirely inward since the ACh response was recorded in isolation from the SK2 component (see below). The evoked membrane current in wild-type IHCs decayed with a complex time course. In IHCs of both Chrna9pto/pto and Chrna9L9/T mice, ACh-evoked membrane current was more reduced than that seen in wild-type mice (i.e., showed reduced desensitization, as previously shown with recombinant α9α10 nAChRs expressed in oocytes [18]). The fraction of current remaining after a 30-s ACh application was 0.29 ± 0.04 in Chrna9pto (n = 10 animals, 15 cells), 0.45 ± 0.05 in Chrna9L9/T (n = 9 animals, 12 cells, p < 0.05), and 0.48 ± 0.06 in Chrna9L9/T (n = 6 animals, 10 cells, p < 0.05).

**Prolonged Synaptic Currents in Mutant IHCs**

Following efferent transmitter release in vitro, spontaneous inhibitory postsynaptic currents (sIPSCs) can be measured in neonatal IHCs. As determined for MOC synapses located on OHCs, the IHC inhibitory postsynaptic currents (IPSCs) result from ion flux through α9α10 nAChRs and calcium-activated SK2 channels [19,20], (Figure 1C). To only measure currents through α9α10 nAChRs, responses were recorded using pipettes filled with KCl-BAPTA (thereby preventing calcium from activating SK2 channels) and 5 mM apamin (an SK channel blocker) in the extracellular solution. As shown in Figure 4A and detailed in Table 1, these “nAChR-only” sIPSCs of mutant mice were dramatically prolonged compared to wild type. There was a 6-fold increase in the tdecay, no difference in trise, and a 3-fold increase in the duration at half amplitude (halfwidth). In addition, the sIPSCs in Chrna9L9/T mice had significantly smaller amplitudes than those of wild-type IHCs. Despite the amplitude reduction, overall charge transfer (as indicated by the area value) of the “nAChR-only” synaptic current was more than doubled in Chrna9L9/T when compared to Chrna9pto IHCs.

When sIPSCs were recorded with KCl-EGTA in the pipette, currents were inward at −90 mV and outward at −40 mV (Figure 4B). As noted for wild-type IHCs ([19,20] and present results), the polarity shift of the sIPSC indicates that SK2 channel gating occurs in the Chrna9L9/T mutant mice. At −90 mV, sIPSCs of Chrna9L9/T mice were dramatically prolonged compared to Chrna9pto with a 4- and 3-fold increase in the tdecay and halfwidth, respectively. At −40 mV, tdecay and halfwidth were 3-fold greater than in wild type, and a slight increase in trise also was observed. The peak current at −40 mV, carried by SK2 channels, was equivalent in Chrna9pto and Chrna9L9/T IHCs. Nonetheless, the smaller amplitude nAChR-only current observed in IHCs of mutant mice activates SK2 channels as effectively as in wild-type IHCs. Indeed, the area under the Chrna9L9/T SK2 current was nearly three times greater than that of the Chrna9pto SK2 current. Presumably, this enhancement results from the increased total calcium ion flux during the prolonged activity of the nAChRs in mutant mice.

As shown in Figure 4C, synaptic activity evoked by 40 mM K+ in IHCs was reversibly blocked by the α9α10 blocker strychnine (300 nM) [13], indicating that the synaptic activity observed in Chrna9L9/T was in fact mediated through mutant α9α10 nAChRs.

**Prolonged Synaptic Currents and Coupling to SK2 Channels in Mutant OHCs**

Since hair cell sIPSCs were infrequently observed, synaptic currents and the effects of exogenous ACh were measured in P10–P11 OHCs using high-potassium buffer (40 mM K+ plus...
EGTA, minus apamin) to depolarize the efferent terminals (Figure 5A and 5B). Under these recording conditions, the synaptic waveform includes current through both the nAChR and associated SK2 channels (entirely inward at $V_{\text{hold}}$ of $-90$ mV since $40$ mM K$^+$ shifts the equilibrium potential of this cation to $-33$ mV). Analysis of waveforms revealed a 4- and 3.5-fold increase in $\tau_{\text{decay}}$ and halfwidth, respectively, and a 0.6-fold reduction in amplitude (Figure 5C and Table 2) in $\text{Chrna9}^{L9T/L9T}$ IPSCs compared to $\text{Chrna9}^{wt/wt}$, much as was observed in IHCs.

Despite a drop in amplitude, the overall charge transfer (as indicated by the area value) was 2-fold larger in $\text{Chrna9}^{L9T/L9T}$ compared to $\text{Chrna9}^{wt/wt}$ OHCs.

Under these recording conditions, synaptic currents reflect ion flux through both nAChRs and associated SK2 channels.

**Table 1. Synaptic Currents in IHCs from $\text{Chrna9}^{wt/wt}$ and $\text{Chrna9}^{L9T/L9T}$ Mice**

<table>
<thead>
<tr>
<th>Currents</th>
<th>nAChR</th>
<th>nAChR + SK2</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{\text{hold}}$</td>
<td>$-82$ mV</td>
<td>$-40$ mV</td>
</tr>
<tr>
<td>Genotype</td>
<td>$\text{wt/wt}$</td>
<td>$\text{L9T/L9T}$</td>
</tr>
<tr>
<td>Amplitude (pA)</td>
<td>$18.4 \pm 0.1$</td>
<td>$11.1 \pm 1.01^a$</td>
</tr>
<tr>
<td>$\tau_{\text{rise}}$ (ms)</td>
<td>$0.8 \pm 0.1$</td>
<td>$0.8 \pm 0.2$</td>
</tr>
<tr>
<td>$\tau_{\text{decay}}$ (ms)</td>
<td>$27.9 \pm 1.0$</td>
<td>$186 \pm 26^c$</td>
</tr>
<tr>
<td>Halfwidth (ms)</td>
<td>$25.0 \pm 1.2$</td>
<td>$79.2 \pm 10.3^b$</td>
</tr>
<tr>
<td>Area (pC)</td>
<td>$0.55 \pm 0.09$</td>
<td>$1.1 \pm 0.1^a$</td>
</tr>
<tr>
<td>Number of animals</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Number of cells</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Number of events</td>
<td>1125</td>
<td>141</td>
</tr>
</tbody>
</table>

$^a p < 0.05$.  
$^b p < 0.01$.  
$^c p < 0.001$.  

wt, wild type.  
doi:10.1371/journal.pbio.1000018.t001
The presence of an ACh-evoked SK2 current was demonstrated directly by application of ACh to Chrna9L9T/L9T OHCs held at different membrane potentials. As shown in Figure 5D, ACh responses were outward at −90 mV, confirming the activation of potassium currents in mutant OHCs.

Increased Cochlear Thresholds in Mutants Can Be Reversed with Strychnine

We examined cochlear responses in Chrna9L9T mutant mice via auditory brainstem responses (ABRs) and distortion product otoacoustic emissions (DPOAEs). Wave 1 of the ABR, which is recorded from scalp electrodes in response to short tone pips, represents the summed activity of cochlear nerve fibers projecting from IHCs (Figure 1B) to the cochlear nucleus, the first central nucleus in the ascending auditory pathway [21]. The DPOAEs are sounds created within the cochlea, amplified by the action of OHCs and propagated through the middle ear back to the ear canal, where they can be measured with a microphone [22]. Given that the generation of DPOAEs requires neither IHCs nor cochlear nerve fibers [23], a comparison of ABRs and DPOAEs can provide insight into the locus of any dysfunction.

As shown in Figure 6A, mean ABR thresholds were elevated by 5–15 dB in both Chrna9wt/L9T (F(1,55) = 4.30, p = 0.043) and Chrna9L9T/L9T (F(1,79) = 7.87, p = 0.06) when compared to Chrna9wt/wt mice. DPOAE thresholds (Figure 6B) were also elevated by 5–15 dB in both Chrna9wt/L9T (F(1,53) = 14.82, p < 0.001) and Chrna9L9T/L9T (F(1,77) = 9.20, p = 0.003). Threshold shifts were larger at frequencies above 16 kHz and were similar in magnitude whether measured by ABRs or DPOAEs, suggesting that all dysfunction can be explained by changes in OHC contributions to cochlear amplification.

Since MOC activity in vivo decreases OHC-based amplification of cochlear responses [2], we asked whether the increased cochlear thresholds might arise from enhanced synaptic currents through mutant α9-containing channels in response to the normal low level of spontaneous MOC activity [24]. To test this hypothesis, we injected mice with strychnine (30 mg/kg, intraperitoneally [i.p.]), a potent blocker of α9x10 channels [13,25]. Indeed, as shown in Figure 7, strychnine improved acoustic thresholds in Chrna9L9T/L9T mice.
mice by almost 9 dB, thus restoring the baseline thresholds to that seen in the wild type.

**Shock-Evoked OC Suppression Is Slowed, Enhanced, and Prolonged in Mutants**

Activation of MOC efferents normally decreases the OHC contribution to cochlear amplification. Thus, the electrical stimulation of the MOC fibers at the floor of the IVth ventricle (Figure 1A, black arrow) results in a decrease in the amplitude of the DPOAEs [25]. To assess MOC function in vivo, DPOAEs were measured before, during, and after a 70-s train of shocks to the olivocochlear bundle. In wild-type mice, DPOAE suppression is seen immediately after shock-train onset (Figure 8A, black arrowhead) and then adapts to a steady state, which is maintained throughout the shock epoch (Figure 8A and 8B). In Chrna9L9T mutant mice, suppression had a much slower onset (Figure 8A, white arrowhead), but continued to grow during the shock train (Figure 8A and 8B) until responses disappeared into the noise floor. To determine the full suppression magnitude, we raised the level of the acoustic stimulus (Figure 8C): peak suppression in Chrna9wt/wt reached approximately 17 dB, whereas for Chrna9T/L9T, the maximum suppression was less than approximately 5 dB. In homozygous mutants, suppression persisted for almost 10 min after the end of the shock train, with a slow recovery to baseline (Figure 8B). Moreover, there is a prominent “overshoot,” which, except for a slower time course, is reminiscent of the post-shock enhancements seen in wild-type mice [25]. As shown in Figure 8A and 8B, responses in Chrna9T/L9T were intermediate between those of homozygous mutant and wild-type mice.

As with wild-type mice [25], suppression could be blocked by strychnine (10 mg/kg), leaving only the postshock enhancement (Figure 9A, grey arrowhead), suggesting that the increased suppression in Chrna9L9T mutants was due to the enhanced activity of 29a10 receptors. With a lower dose of strychnine (3 mg/kg), suppression in Chrna9T/L9T resembled the normal Chrna9T/L9T response (i.e., reduced suppression magnitude but with rapid onset and decay; Figure 9B, grey arrowhead).

**Discussion**

The present study demonstrates that substitution of a single amino acid in transmembrane region 2 of the 29 nAChR subunit (L9T) enhances nAChR function in cochlear hair cells (i.e., decreased ACh EC50 and slowed receptor desensitization rate), increases synaptic efficacy, and dramatically strengthens cochlear suppression in vivo. More importantly, it shows that the MOC efferent system inhibits cochlear...
To reveal full suppression magnitude, we raised primary levels until preshock DPOAEs were 25 dB above the noise floor: peak suppression in primary levels were adjusted to produce a DPOAE approximately 15 dB above the noise floor: suppression is so strong in mutant ears that the DPOAEs are driven into the noise. WT, wild type.

The Hair Cell as a Model System to Study the Nicotinic Synapse

Understanding neurotransmission mediated by neuronal nicotinic receptors is a fundamental challenge in neuroscience, since decline, disruption, or alteration of nicotinic cholinergic mechanisms in the central nervous system contributes to dysfunctions such as epilepsy, schizophrenia, Parkinson's disease, autism, dementia with Lewy bodies, Alzheimer's disease, and addiction [10]. Although fast, direct nicotinic synaptic activity drives neurotransmission in autonomic ganglia, only rare cases of fast nicotinic transmission mediated by neuronal nAChRs have been reported in the mammalian brain. Because cholinergic neurons in the brain are usually loosely distributed and often sparsely innervate broad areas, it is experimentally difficult to stimulate a large number of those neurons and to record selectively from their postsynaptic targets. Indeed, it is likely that authentic fast nicotinic transmission is present at low densities in more neuronal areas than the few that have been reported [10]. Furthermore, the variable transcription of genes coding for nAChR subunits and the possible combinatorial assembly of these subunits produces a wide structural diversity of receptor types. This imposes additional challenges for studying native synaptic neuronal nAChRs [31].

Thus, the cholinergic synapse between MOC terminals and cochlear hair cells provides a valuable model for the study of fast neurotransmission mediated by nAChRs of known composition [12,13]. Targeted mutations can be introduced and the phenotypic consequences can be analyzed at the synaptic, whole-organ, and systems level. When analyzed at the level of nAChR function, the α9 L9T mutant mice reproduce what has been previously described for the recombinant receptor expressed in oocytes, i.e., a decrease in EC50 for ACh and a reduced desensitization kinetics [18]. Both these effects probably derive from the fact that the L9T position of nAChRs is critical for channel gating [32] and that hydrophilic substitutions at this position lead to increased mean open times [18,33]. Moreover, these changes in channel gating properties translate into increased synaptic efficacy, as seen from the prolonged synaptic currents observed in IHCs and OHCs of mutant mice. In addition, the inhibitory signature of the efferent synapse is conserved, since the nAChR currents remain coupled to the associated SK2 currents, which were also substantially prolonged. In fact, the increase in overall charge transfer during sIPSCs between wild-type and knockin mice was more pronounced when the secondary SK2 currents were measured, than for the nAChR-only currents (Table 1), pointing to an additional amplified step, possibly calcium-induced calcium release from the nearby synaptic cistern [34]. Finally, the finding that the L9T mutation does not lead to hair cell death, is distinct from effects of similar mutations in α4 and α7 nAChR subunits, which lead to death of substantia nigra dopaminergic neurons [35] and apoptotic cell death throughout the somatosensory cortex [36], respectively, most likely due to Ca2+ excitotoxicity. The observation that α9α10 receptors are indeed highly permeable to Ca2+ [37,38] points toward an efficient Ca2+ buffering system in hair cells. In fact, proteinaceous calcium buffers (e.g., parvalbumin-β) are expressed in OHCs at high levels, similar to those found in skeletal muscle [39].

Functional Consequences of Enhanced nAChR Gating

The main peripheral effect of the MOC activity is to inhibit cochlear responses by decreasing the gain of the cochlear amplifier [2]. MOC neurons comprise the effector arm of a sound-evoked negative feedback system that, in a quiet environment, normally has little effect on cochlear sensitivity,
since MOC neurons have low levels of spontaneous activity and do not respond to sound until levels exceed threshold by 15–20 dB [24]. The fact that the baseline threshold elevation in L9'T mutants can be "rescued" via strychnine, the potent a9a10 nAChR blocker, suggests that it arises from an enhancement of cholinergic effects on OHCs. Therefore, compared to Chrn9 and Chrn10 knockouts, in which baseline cochlear thresholds were normal [40,41], the present knockin strategy reveals cholinergic MOC effects under resting conditions. This baseline inhibition could arise from an increased sensitivity of mutant receptors to normal low levels of spontaneous ACh release from MOC terminals or to the increased probability of spontaneous channel openings of L9'T mutant receptors in the absence of ACh [18]. The appearance of spontaneous channel openings, as described for the mutant receptors [18], may also explain how low-dose strychnine can speed the onset kinetics of the in vivo response in mutant mice (Figure 9B), i.e., by restoring mutant channels to the closed state, from which rapid ACh-mediated channel openings can occur.

Hair cell recordings from the L9'T mutant mice in the recent study show (1) ACh-evoked currents with a greatly reduced desensitization rate, and (2) spontaneous miniature synaptic currents with slower activation and decay kinetics. These changes in ACh response kinetics provide likely explanations for some aspects of the electrically evoked suppression of DPOAEs. The doubling of onset time for the ACh-evoked SK2 current in mutant hair cells (Table 1) must contribute to the slowed onset of MOC-mediated suppression in vivo, by reducing linear summation of postsynaptic effects. However, the slowing of suppression onset is so dramatic (<1 s in wild type vs. >20 s in mutant mice) that other possible factors, e.g., alterations of release probability or slowed facilitation in the efferent terminals [42], might also contribute to this delayed time course.

In addition to rising more slowly, MOC-mediated cochlear suppression was also larger and longer lasting in L9'T mutant mice. The normal adaptation of suppression during continuous MOC activation was absent in the presence of slowly desensitizing a9L9'T mutant receptors, consistent with the idea that receptor desensitization is a key factor in the decay of the wild-type response after shock-train onset. However, the dramatic prolongation of cochlear suppression after shock-train offset (500 s in mutants vs. <5 s in wild types) is too large to be explained only by the 2- to 3-fold prolongation of synaptic currents observed in the mutant hair cells. Another contributing factor may be the observation that choline, the metabolite produced when acetylcholinesterase degrades ACh released at the synapse, is a full agonist of the mutant a9a10 receptor, and only a weak partial agonist of the wild-type receptor [18]. Thus, in vivo, the suppression can only decay after choline is taken up by the synaptic terminal or otherwise diffuses away. Treatment with a low dose of the a9a10 antagonist strychnine (3 mg/kg) supports this interpretation. This treatment completely abolished the prolonged postshock suppression in mutant mice (and unmasked a fast onset). Since choline has 4-fold lower affinity for a9L9'T mutant receptors than ACh [18], a low concentration of strychnine may be differentiating the effects of the two agonists (i.e., blocking the effects of the agonist with a lower potency, choline, but leaving unchanged the effects of the high-affinity agonist, ACh), as well as modulating the resting level of desensitization. Beyond the possibility of metabolite activation, extended inhibitory effects could involve more than changes in membrane conductance. For example, it has been shown that exposure to ACh alters the stiffness and motility of OHCs isolated from the gerbil cochlea over tens of minutes [43]. Stiffness and motility of OHCs depends, at least in part, on the motor protein prestin, a molecule that has selectively evolved in mammals to subserve somatic electromotility and amplification [44,45]. Thus, it remains to be determined whether prolonged Ca2+ influx through mutated a9a10 receptors leads to changes in prestin structure or function resulting in prolonged MOC efferent effects.

The presence of mutant a9L9'T receptors, designed to increase the magnitude of MOC effects on OHCs, also increased the protective action of the MOC system in vivo.
This is consistent with previous work showing that overexpression of wild-type α9 channels, which more modestly increased the magnitude of MOC-mediated DPOAE suppression, also increased the resistance of the ear to acoustic injury [26]. Prior in vivo studies of electrically evoked MOC activity have described both fast and slow effects of ACh on cochlear neural responses [46,47] and cochlear mechanical vibrations [48]. The fast suppression, with an onset time course of approximately 100 ms, arises from the increased K⁺ conductance in neighboring SK2 channels and the effects of the resultant OHC hyperpolarization on the magnitude of electromotility and thus on cochlear vibration amplitude. The slow suppression, with an onset time course of approximately 10 s, may require a wave of calcium-induced calcium release along the OHC membrane and appears to reflect a change in OHC stiffness [48] that also reduces cochlear mechanical vibrations [49]. The fast suppression overexpression of SK2 channels does not increase resistance to acoustic injury, although as with α9 overexpression, it also increases the magnitude of MOC-mediated DPOAE suppression, suggests that it is the slow effects of ACh that are responsible for its protective action, rather than the hyperpolarization-induced decrease in electromotility.

There are two fundamentally different ways in which slow effects of α9 activation could reduce acoustic injury: (1) by reducing mechanical vibration of the sensory epithelium, or (2) via intracellular modifications of OHCs arising as downstream effects of the calcium entry through α9 receptors. The further observation that in the α9L9′T mutants, protection was seen only for exposures intense enough to produce irreversible damage and permanent threshold shifts, and not in less traumatic exposures producing only reversible changes, argues against a general reduction of vibration amplitude as the underlying mechanism. Most of the permanent threshold elevation underlying this type of noise exposure arises from damage to the hair cell stereocilia bundles, including disarray loss and/or fusion of these modified microvilli [50], which house the mechanoelectric transduction channels. Further insight into the mechanisms linking calcium entry through nAChRs and protection from acoustic injury will require a clearer delineation of the molecular events leading up to this type of noise-induced stereocilia damage.

Conclusions

This work shows that a point mutation in the hair cell's nAChR produces dramatic prolongation of efferent MOC inhibitory effects at both cellular and systems levels. This alteration provides enhanced protection from permanent acoustic trauma, indicating that cholinergic synaptic feedback is not only necessary, but sufficient for this effect. In addition, the enhanced activity of the modified nAChR revealed a novel tonic inhibitory effect, raising baseline acoustic thresholds over those of wild-type littermates, confirming the inhibitory nature of the MOC efferent system. Thus, this α9L9′T knockin presents new insights into the cellular mechanisms of cholinergic inhibition, as well as a promising model in which to probe the functional role of MOC efferents. In addition, the hair cell's efferent synapse, much like the neuromuscular junction, can provide insights into cholinergic signaling, and promises to be an equally informative model for studying activity-dependent synaptic function.

Materials and Methods

Genetic engineering and genotyping of Chrna9L9′T knockin mice. A HindIII-NotI (the latter derived from the construction of the library) restriction endonuclease fragment of approximately 9.500 bp encoding Chrna9 exons 1–4 (see GenBank accession number NT_039305.7 and Figure 2A) was obtained from a mouse strain 129S4/SvJae genomic DNA library (kindly provided by Dr Bernhard Better, University of Basel) and subcloned into the vector pKO-Select DT (Lexicon Genetics). A 2-kbp neomycin resistance cassette flanked by two loxp sites (loxP-neo-loxP) was inserted in the NcoI restriction site located between exons 3 and 4. The Chrna9L9′T mutation (+) introduced via site-directed mutagenesis using the QuickChange Site-Directed Mutagenesis kit (Stratagene) and amplifiers Asense (5′-CTCTGGGAGTGACCATCCTAAaggggcatGAGTCGCTATTTCCAGC-3′) and Aantisense (5′-GGTGAATAACAGTCAAGTGGCgggtTAGGATGTCACCCAGAG-3′). This targeting vector was used to electroporate 129S4/SvJae embryonic stem (ES) cells, and homologous recombinants were obtained following gentamicin (G418) selection and Southern blot hybridization analyses. Genomic DNA was purified from G418-resistant ES cell clones, digested with HindIII and KpnI, electrophoresed on 0.8% agarose, and hybridized to a 32P-labeled DNA 902-bp SacII-KpnI fragment probe prepared from the DNA fragment shown in Figure 2A. Based on the sequence of the mouse Chrna9 subunit gene (see GenBank accession number NT_039305.7), wild-type ES cell DNA yielded a fragment of 13,800 bp, whereas ES cells that have undergone homologous recombination yielded a 7,300-bp fragment (see Figure 2B). Transfection of the linearized targeting vector into murine ES cells resulted in the insertion of the L9′ mutation into the α9 nAChR subunit. The frequency of homologous recombination events in ES cells was 42%. Six independent ES cell lines carrying the mutation were injected into blastocysts to generate germline chimeric male and female progeny mice were backcrossed to strain C57BL/6J females, and the resulting offspring were intercrossed to generate α9L9′T knockin mice.

This work shows that a point mutation in the hair cell's nAChR produces dramatic prolongation of efferent MOC inhibitory effects at both cellular and systems levels. This alteration provides enhanced protection from permanent acoustic trauma, indicating that cholinergic synaptic feedback is not only necessary, but sufficient for this effect. In addition, the enhanced activity of the modified nAChR revealed a novel tonic inhibitory effect, raising baseline acoustic thresholds over those of wild-type littermates, confirming the inhibitory nature of the MOC efferent system. Thus, this α9L9′T knockin presents new insights into the cellular mechanisms of cholinergic inhibition, as well as a promising model in which to probe the functional role of MOC efferents. In addition, the hair cell's efferent synapse, much like the neuromuscular junction, can provide insights into cholinergic signaling, and promises to be an equally informative model for studying activity-dependent synaptic function.
and flanking regions of the targeted allele in neo-deleted mice were confirmed by DNA sequencing.

The Chrna9L9/T mutant allele has been maintained in congenic FVB.129P2-Pde6b-/+ Tyrc-chAntJ (stock number 004828; Jackson Laboratory) strain. C57BL/6J mice develop a marked and progressive late-onset hearing loss characterized by cochlear degeneration. The background hearing loss seen in the C57BL/6J mice was lost upon backcrossing to congenic FVB.129P2-Pde6b-/+ Tyrc-chAntJ stock for up to 5 generations (i.e., N4–N5).

Routine genotyping of Chrna9 mice was performed using tail biopsy tissue DNA samples (Wizard Genomic DNA Purification kit; Promega), amplifiers A9LOPX.1 (5′-TAC CGG CTA TCC TGC AGA CAG AGC-3′) and A9LOXP.2 (5′-AGG AGC GAG CAG AGG TCA TAA AAG GTG AGG-3′) and FastStart PCR Master Kit with buffer B (Epicentre) as described by the manufacturer. PCR cycle parameters were: 95 °C, 0.5 min; 55 °C, 1.0 min; and 72 °C, 2 min for a total of 35 cycles. Reaction products were electrophoresed on 1.5% agarose, stained with ethidium bromide, and photographed.

The PCR fragment length for the wild-type Chrna9 allele is 203 bp and 269 bp for the mutant allele (see Figure 2C). In each breeding pair, the PCR fragment length for the wild-type Chrna9 (5′-CTCTCTGACTTCATTGAAGACG-3′) and A9LOXP.2 (5′-AGG AGC GAG CAG AGG TCA TAA AAG GTG AGG-3′) and L9T3′ (5′-CCGCACACATCCTAGGGTCGAT-3′) (Figure 2D).

Electrophysiological recordings from cochlear hair cells. Mice were sacrificed by decapitation. All experimental protocols were carried out in accordance with the American Veterinary Medical Associations’ AVMA Guidelines on Euthanasia (June 2007). Apical turns of the organ of Corti were excised from mice and incubated in high Ca2+ media containing 1 mg/ml Hgs and P10–P11 for OHGs, and used within 3 h. Day of birth was considered postnatal day 0 (P0). Cochlear preparations were mounted under a Leica DMLS microscope (Leica Microsystems) and viewed with differential interference contrast (DIC) using a 40× water immersion objective and a Hamamatsu C7900–50 camera with contrast enhancement (Hamamatsu). Methods to record from IHCs and OHGs were essentially as described previously [17,19].

Briefly, hair cells were identified visually with the 40× objective and during recordings, by the size of their capacitance (7 to 12 pF), by their responses to IHCs’ during recordings, by the size of their capacitance (7 to 12 pF), by their responses to a single stimulus, and by their specific intracellular current (nAChR-only). In addition, in the latter condition, the SK current was abolished by the application of 10 mM 9-aminoacetocetic acid (BAPTA) (KCl-BAPTA saline). The latter protocol was used where indicated, because many mutants experienced audiogenic seizures as soon as the high-level noise was turned on. The threshold for acoustic–cortical seizures was defined as the f1 level required to produce a DPOAE amplitude of ≤ 0.3 dB below that of the noise floor at 56, followed by a series of 70 contiguous periods in which DPOAE amplitudes were measured with simultaneous shocks to the MOC bundle and additional periods during which DPOAE amplitudes were measured after the termination of the shock train.

Olivocochlear assay. Mice were anesthetized with urethane (1.20 g kg−1) and xylazine (20 mg kg−1). A posterior craniotomy and partial cerebellar aspiration were performed to expose the floor of the IVth ventricle. To stimulate the MOC bundle, shocks (monophasic pulses, 150–μs duration, 2060) were applied through fine silver wires (0.4-mm spacing) placed along the midline, spanning the olivocochlear decussation. Shock threshold for facial twitches was determined, muscle paralysis induced with 2% thiopental (2.5 mg kg−1 i.p.), and the animal connected to a respirator via a tracheal cannula.

Shock levels were raised to 6 dB above the threshold of the noise floor before either recording. The MOC suppression assay, f2 level was set to produce a DPOAE 10–15 dB or 20–25 dB greater than the noise floor. To measure MOC effects, repeated measures of DPOAE amplitudes were obtained following an 8–16-kHz octave band noise presented at 100 dB SPL (for temporary injury). For the higher level exposure, animals were anesthetized and xylazine, exactly as for the MOC suppression assay, was used to produce 10–15 (n = 55) dB of acoustic trauma, because many mutants experienced audiogenic seizures as soon as the high-level noise was turned on. The exposure stimulus was generated by a custom white-noise source, filtered (Brickwall Filter with a 60-dB-lowslope), amplified (Pound power amplifier), and delivered to the SPL completely through an exponential horn fitted securely to a hole in the top of a reverberator box. Sound exposure levels were measured at four positions within each cage using a 0.25″ Bruel and Kjaer condenser microphone: sound pressure was found to vary by less than 0.5 dB across these measurements.

Data analysis. Statistical analyses of all, as well as a two-way ANOVA with repeated measures, was performed with R version 2.8.0. A p < 0.05 was selected as the criterion for statistical significance. Mean values are quoted as means ± the standard error of the mean (S.E.M.).

Materials. ACh chloride, strychnine HCl, Na2ATP, BAPTA, and all
other reagents were from Sigma Chemical. EGTA and Na2ATP were dissolved at the moment of preparing the intracellular solutions.

**Animal welfare.** All experimental protocols were carried out in accordance with the National Institutes of Health guide for the care and use of laboratory animals as well as Instituto de Investigaciones en Ingenierı´a Genetı´ca y Biologı´a Molecular (INGEBI), Tufts University, and Massachusetts Eye and Ear Infirmary Institutional Animal Care and Use Committee (IACUC) guidelines, and best practice procedures.

**Supporting Information**

Figure S1. Morphology of the Cochlear Duct Is Normal in Mutant Ears

Gross histological assessment via plastic sections of osmium-stained Chrna9L9 mice cochlea showed no morphological changes when compared to Chrna9wt. Sections through the middle turn are shown. Black and grey arrows in Chrna9wt indicate OHCs and IHCs, respectively. Histological procedures were performed as described in Text S1.

Found at doi:10.1371/journal.pbio.1000018.s001 (238 MB TIF).

**Figure S2.** Voltage-Dependent K+ Currents from IHCs and OHCs (A) Voltage-dependent currents obtained in P6–P10 IHCs by holding the cell at -80 mV and stepping the voltage to 80 mV in 10-mV increments (200-ms duration). Representative traces are shown for the three genotypes as well as for averaged steady state current-voltage curves (Chrna9wt: n = 10 animals, 13 cells; Chrna9wt: n = 9 animals, 13 cells; and Chrna9wt: n = 14 animals, 17 cells); (B) same as in (A), but for P10–P14 OHCs (Chrna9wt: n = 2 animals, 3 cells; and Chrna9wt: n = 6 animals, 8 cells). Found at doi:10.1371/journal.pbio.1000018.s002 (225 KB TIF).

**Figure S3.** Dose-Response Curve for Strychnine Blockade of Cholinergic OC Effects on DPOAE Thresholds

Each point shows data from different animals extracted from runs such as those shown in Figure 8 (f0 = 22.6 kHz). All data were collected 60 min after strychnine injection. Although thresholds in wild-type mice slightly rise with strychnine dosage, thresholds in knockin mice improve by as much as 8 dB. Found at doi:10.1371/journal.pbio.1000018.s003 (117 KB TIF).

**Table S1.** Analysis of Gene Expression by Quantitative PCR

<table>
<thead>
<tr>
<th>Chrna9: gene encoding the α9 nAChR subunit</th>
<th>Chrna10: gene encoding the α10 nAChR subunit</th>
<th>Kenm1: gene encoding the SK2 channel</th>
<th>Ryr1: gene encoding the ryanodine receptor 1</th>
<th>Ryr2: gene encoding the ryanodine receptor 2</th>
<th>Ryr3: gene encoding the ryanodine receptor 3</th>
<th>Kenm1: gene encoding the calcium-activated potassium channel (BK) β1-subunit</th>
<th>CaV1.3: gene encoding the voltage-gated calcium channel α1-subunit</th>
<th>Myo7a: gene encoding myosin VIIa</th>
<th>C1q2: threshold cycle of sample; C1m: threshold cycle of Myo7a. Results shown are the mean ± S.E.M of three experiments per group, each performed in triplicates. The protocol for quantitative PCR was as indicated in Text S2.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chrna9wt/wt; n=14 animals, 17 cells</td>
<td>Chrna9wt/wt; n=2 animals, 3 cells</td>
<td>Chrna9wt/wt; n=6 animals, 8 cells</td>
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**Author contributions.** JT, SFM, JAB, EK, DEV, JB, MCL, PAF, and ABE conceived and designed the experiments. JT, SFM, JS, and JB performed the experiments. JT, SFM, JAB, DEV, MCL, PAF, and ABE analyzed the data. SFM, MCL, PAF, and ABE wrote the paper.

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**Competing interests.** The authors have declared that no competing interests exist.

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