Activity-dependent regulation of prestin expression in mouse outer hair cells

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Activity-dependent regulation of prestin expression in mouse outer hair cells. J Neurophysiol 113: 3531–3542, 2015. First published March 25, 2015; doi:10.1152/jn.00869.2014.—Prestin is a membrane protein necessary for outer hair cell (OHC) electromotility and normal hearing. Its regulatory mechanisms are unknown. Several mouse models of hearing loss demonstrate increased prestin, inspiring us to investigate how hearing loss might feedback onto OHCs. To test whether centrally mediated feedback regulates prestin, we developed a novel model of inner hair cell loss. Injection of diphtheria toxin (DT) into adult CBA mice produced significant loss of inner hair cells without affecting OHCs. Thus, DT-injected mice were deaf because they had no afferent auditory input despite OHCs continuing to receive normal auditory mechanical stimulation and having normal function. Patch-clamp experiments demonstrated no change in OHC prestin, indicating that loss of information transfer centrally did not alter prestin expression. To test whether local mechanical feedback regulates prestin, we used TectaC1509G mice, where the tectorial membrane is malformed and only some OHCs are stimulated. OHCs connected to the tectorial membrane had normal prestin levels, whereas OHCs not connected to the tectorial membrane had elevated prestin levels, supporting an activity-dependent model. To test whether the endocochlear potential was necessary for prestin regulation, we studied TectaC1509G mice at different developmental ages. OHCs not connected to the tectorial membrane had lower than normal prestin levels before the onset of the endocochlear potential and higher than normal prestin levels after the onset of the endocochlear potential. Taken together, these data indicate that OHC prestin levels are regulated through local feedback that requires mechanoelectrical transduction currents. This adaptation may serve to compensate for variations in the local mechanical environment.

SOUND-INDUCED VIBRATIONS of the organ of Corti deflect the sensory hair cell stereociliary bundle, creating a receptor potential within the soma. In response, outer hair cells (OHCs) generate force, a process termed electromotility (Brownell et al. 1985). These forces amplify the cochlear traveling wave and increase organ of Corti vibration, producing the exquisite auditory sensitivity and frequency selectivity characteristic of normal mammalian hearing. Prestin (SLC26A5), a tetrameric protein present within the lateral wall plasma membrane of OHCs, is necessary for electromotility (Cheatham et al. 2004; Dallos et al. 2008; Hallworth and Nichols 2012; Liberman et al. 2002; Wang et al. 2010). Prestin is often modeled as having two states so that voltage-dependent stochastic transitions fit a Boltzmann function and produce electromotility (Dallos et al. 1993; Iwasa 1994). These transitions are associated with charge movement across the membrane that can be measured as a voltage-dependent non-linear capacitance (NLC) under patch-clamp recording conditions (Kakehata and Santos-Sacchi, 1995 and 1996; Santos-Sacchi 1991; Santos-Sacchi et al. 1998a). Thus, measuring the NLC is a common way of assessing the amount of functional prestin within an OHC (Abe et al. 2007; Oliver and Fakler 1999).

There are many ways to modulate the voltage dependence of force production by prestin protein within the plasma membrane, such as changing the surrounding lipid environment, intracellular Ca2+ level, membrane tension, membrane potential, and ionic composition of the intracellular and extracellular compartments (Kakehata and Santos-Sacchi 1995; Keller et al. 2014; Rajagopalan et al. 2007; Ratnanather et al. 1996; Santos-Sacchi et al., 1998b and 2001; Wu and Santos-Sacchi 1998). However, the basic mechanisms of how prestin levels within the plasma membrane are regulated remain unclear. We know that prestin has an extremely high density within the lateral wall plasma membrane. It has a charge density of ~6,000–10,000 e−/µm2 (where e− is the electron charge) (Belyantseva et al. 2000; Mahendrasingam et al. 2010), and an estimated 60–75% of the lateral wall surface area is composed of intramembranous particles, thought to be composed of or include prestin (Forge 1991; Kumano et al. 2010; Wang et al. 2010). In addition, prestin−/− OHCs transfected with prestin driven by the constitutively active cytomegalovirus promoter have a similar prestin density to untransfected wild-type OHCs (Xia et al. 2008). These data suggest that prestin production is not limited at the level of transcription but argue instead that mechanical considerations physically limit the amount of prestin within the plasma membrane.

However, there is growing evidence that prestin levels can increase in states of hearing loss. For example, prestin mRNA expression is increased after salicylate administration (Yu et al. 2008), after noise exposure (Chen 2006; Mazurek et al. 2007; Xia et al. 2013), and in the presence of a malformed tectorial membrane (Liu et al. 2011; Xia et al. 2010). These observations suggest the possibility that hearing loss stimulates OHCs to upregulate prestin, perhaps in an effort to compensate for decreased hearing sensitivity. However, all of these previously published reports created hearing loss in animals by affecting OHCs in some way. Thus, it remains unclear whether higher brain centers recognize a state of hearing loss and provide efferent feedback to residual OHCs via the medial olivocochlear bundle (Fig. 1, A and B). Alternatively, signaling could occur locally within the epithelium to regulate prestin levels through a direct reduction in the OHC stereociliary bundle stimulation and/or reciprocal synapses that have been found in type II nerve fibers that innervate multiple OHCs within a row (Francis and Nadol Jr. 1993; Spoendlin 1969). In the present study, we sought to clearly differentiate between efferent feedback and local feedback as regulators of prestin level using two different mouse models that allowed us to isolate the...
source of the feedback. Our findings indicate that prestin is regulated locally within the epithelium in an activity-dependent manner.

**METHODS**

The Institutional Animal Care and Use Committee of Stanford University approved the study protocol.

_Diptheria toxin mouse model_. This mouse model has not been previously published. We used 5-wk-old wild-type CBA/CaJ mice (stock no. 000654, The Jackson Laboratory, Bar Harbor, ME). These mice have stable auditory thresholds from postnatal day (P)12 to P21 (Ohlemiller et al. 2010). Intraperitoneal injections of diptheria toxin (DT) (no. 15042B1, List Biological Laboratories, San Jose, CA), each at a dosage of 50 ng/g, were administered for 3 days in a row. Uninjected mice of the same age were used as controls. DT-injected mice with diptheria toxin (DT inj) demonstrated severe IHC loss and thus lacked an afferent neural response. OHC stimulation was unaffected. _Caja_C1509G_ and _Caja_C1509G_ mice (top). None of the OHCs are stimulated in _Caja_C1509G_ mice (bottom).

_Fig. 1. Experimental concept and mouse models. A_, illustration of a normal organ of Corti. The tectorial membrane (TM), inner hair cell (IHC), outer hair cell (OHC) rows (1, 2, and 3), basilar membrane (BM), afferent auditory nerves (blue arrow), medial olivocochlear efferent nerves (green arrow) are shown. The TM stimulates OHC stereociliary bundles in all three rows of OHCs in control mice. _B_, potential mechanisms of prestin regulation. OHCs amplify the vibration of the organ of Corti in synchrony with its stereociliary bundle stimulation. This drives IHC stimulation and an afferent auditory response. Higher brain centers could then modulate efferent neural activity, whicheffects a change in OHC prestin levels. We broke this feedback loop using mice with IHC loss. Alternatively, OHC bundle stimulation could directly regulate prestin within individual OHCs or along a row via reciprocal synapses in type II nerve fibers. We selectively blocked OHC bundle stimulation using _Caja_C1509G_ mice. _C_, mice injected with diptheria toxin (DT inj) demonstrated severe IHC loss and thus lacked an afferent neural response. OHC stimulation was unaffected. _D_, _Caja_C1509G_ mice had altered TM anatomy. The TM only stimulates first-row OHCs in _Caja_C1509G_ mice (top). None of the OHCs are stimulated in _Caja_C1509G_ mice (bottom).
not detectable at 80 dB SPL, we then arbitrarily defined the threshold at that frequency to be 80 dB SPL.

DPOAEs were measured by placing a probe tip microphone (type 4182, Bruel & Kjaer) in the external auditory canal. The frequency response of this microphone was measured using a free-field microphone (type 4939, Bruel & Kjaer) that has a flat frequency response out to 100 kHz and compensated for in the software. The stimuli used to elicit DPOAEs were two sine wave tones 1-s in duration with different frequencies, where frequency 2 (F2) = 1.22 × frequency 1 (F1). F2 was varied from 4 to 70 kHz, and the two tones were given at identical intensities ranging from 20 to 80 dB in 10-dB steps. The magnitude of the acoustic signal detected from the microphones was measured at the 2 × F1 – F2 frequency. DPOAE responses for each frequency were interpolated, and the threshold was determined to be when the DPOAE magnitude was both >5 dB SPL and >2 SD from the noise floor. If a DPOAE signal was not detectable at 80 dB SPL, we then arbitrarily defined the threshold at that frequency to be 80 dB SPL.

**Basilar membrane tuning curves.** Basilar membrane vibrations were measured using a custom-built optical coherence tomography system using previously described techniques (Gao et al., 2013 and 2014; Lee et al., 2015). Briefly, mice were anesthetized with a ketamine-xylazine mixture and secured in a head holder, and the bulla was surgically opened. The mouse was oriented to view the region of the cochlea one-half turn down from the helicotrema. By imaging noninvasively through the otic capsule bone, vibratory data were collected from the midportion of the basilar membrane over the frequency range from 2 to 13 kHz. Stimulus intensities ranged from 10 to 80 dB SPL. Vibratory magnitude responses below the noise floor of 0.11 nm were not analyzed. Q10 dB values were calculated using the vibratory data collected with the 10 dB SPL stimulus as the characteristic frequency (the frequency of maximum vibratory amplitude) divided by the bandwidth 10 dB down from the peak. Cochlear gain was calculated by dividing the maximum vibratory amplitude in the active cochlea (living) by that of the passive cochlea (dead) using 10 dB SPL stimuli. Since we could not measure the response of the passive cochlea with a 10 dB SPL stimulus because it was below the noise floor of our system, we used the vibratory response of the basilar membrane to the 80 dB SPL stimulus and scaled it down linearly by the noise floor of our system, we used the vibratory response of the basilar membrane to the 80 dB SPL stimulus and scaled it down linearly by

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\text{Q}_{10 \text{ dB}} = \text{Max} \text{V} / \text{Lin V}_{10 \text{ dB}}
\]

where \(V\) is voltage. Linear capacitance (\(C_{\text{Lin}}\)) was calculated as the average capacitance at \(-150\) and \(+120\) mV. NLC was then determined using a Nanodrop ND1000 spectrophotometer. Samples not meeting concentration and purity thresholds were discarded. cDNA was then generated using iScript Reverse Transcription Supermix for quanitative real-time PCR (Bio-Rad). A control reaction with water-immers-

**Whole mount preparations.** Whole mount preparations of the cochlear epithelium were prepared as previously described (Xia et al., 2013). Briefly, cochleae were excised, fixed in 4% paraformaldehyde at room temperature for 1 h, and immersed in 0.5 M EDTA overnight. Cochleae were then rinsed with PBS containing 0.1% Triton X-100 three times for 5 min each. The entire organ of Corti was dissected out of the cochlea under a microscope. Immunolabeling was performed by first blocking the cochlea with 4% donkey serum (017-000-121, Jackson ImmunoResearch Laboratories, West Grove, PA) in PBS in TWEEN 20 (PBST) for 1 h at room temperature before incubation with the primary antibody overnight at 4°C. Cochleae were washed four times with PBST, incubated with secondary antibody at room temperature for 1 h, and rinsed with PBS. The primary antibody was rabbit anti-myosin VIIA (1:200, Proteus Biosciences, Ramona, CA) diluted in PBST. Secondary antibody was Alexa Fluor 568 donkey anti-rabbit (1:500, Invitrogen, Grand Island, NY) diluted in PBST. After being labeled, cochleae were washed with PBS and mounted in antifade fluorescence mounting medium (DAKO, Carpinteria, CA) and coverslipped. A confocal Zeiss LSM5 Pascal microscope system with a ×20/0.5 EC Plan-NEOFLUAR objective was used to image hair cells.

**Statistical analysis.** All data were analyzed with Microsoft Excel (Microsoft Office 2007). Plots and figures were created with SigmaPlot (11.0, Systat Software, San Jose, CA), Adobe Photoshop CS6 (Adobe Systems, San Jose, CA), and Adobe Illustrator CS6 (Adobe Systems). Statistical significance was determined using either a non-

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\text{CT,control) relative quantification method.}
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\[
\text{d} = \frac{\text{Q}_{\text{max}} / e^{-}}{\text{C}_{\text{Lin}}} / \text{C}_{\text{Conv}}
\]

where \(d\) is charge density or the number of electrons moved per square micrometer of cell surface area, \(e^{-}\) is the electron charge \((1.602 \times 10^{-19})\), and \(C_{\text{Conv}}\) is the specific membrane capacitance of 0.008 pF/μm². Fakler 1999) as follows:

\[
\text{C}(V) = C_{\text{Lin}} + \frac{Q_{\text{Max}}}{1 + \text{exp}[V - V_{1/2}]} \left[1 + \text{exp}[V - V_{1/2}]/\alpha\right]^{2}
\]

\text{where C is capacitance and V is voltage. Linear capacitance (C_{\text{Lin}}) was calculated as the average capacitance at -150 and +120 mV. NLC was calculated as the difference between peak capacitance and linear capacitance. Charge density was then calculated using the specific membrane capacitance previously determined for mouse OHCs (Abe et al., 2007). The following equation was used:}

\[
\text{where} \quad \text{d} = \frac{\text{Q}_{\text{Max}} / e^{-}}{C_{\text{Lin}} / C_{\text{Conv}}}
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\[
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paired two-tailed Student’s t-test (for comparisons of two groups), one-way ANOVA followed by a nonpaired two-tailed Student’s t-test for significant findings (for comparison of more than two groups), or two-way ANOVA (for multiple comparisons among two or more groups). P values of <0.05 were considered significant. Baseline currents are reported as means ± SD; all other data are presented as means ± SE.

RESULTS

DT causes inner hair cell death but leaves OHCs intact. We first studied a mouse that has hearing loss due to inner hair cell (IHC) loss (Fig. 1C) to investigate whether hearing loss, as detected by higher brain centers, modulates efferent feedback to regulate prestin. Thus, any efferent feedback pathways that regulate prestin should be activated if hearing loss upregulates prestin. This new mouse model was discovered serendipitously while working on another research project using a transgenic mouse where the DT receptor gene was targeted to a different cell population. As a control, we injected adult wild-type CBA mice with DT (referred to henceforth as “DT-injected mice”). In these DT-injected mice, we found a dramatic loss of IHCs 3 days after the injection (Fig. 2, A and B). OHCs were only minimally affected. We counted IHCs and OHCs from three to five whole mount preparations from the middle turn of the cochlea from three control and three DT-injected mice. Each preparation contained 16–22 IHCs and 45–67 OHCs in control mice. There was a 69% loss of IHCs (P < 0.001 by t-test) and a 2% loss of OHCs (P = 0.03 by t-test) 3 days after DT injection (Fig. 2C).

We then studied cochlear function in a cohort of CBA mice by measuring ABRs and DPAOEs from the left ears of 12 mice before DT injection and again 3 days after the injection (Fig. 2D). After DT injection, mice had no measurable ABRs to equipment limits (P < 0.01 by two-way ANOVA). In contrast, DPOAE thresholds after injection were no different from control mice. We then calculated Q10 dB and cochlear gain in 2E responses, and the responses became linear postmortem (Fig. 2E). To directly assess whether prestin levels were affected in DT-injected mice, we patch clamped 17 first-row OHCs (8 DT-injected OHCs and 9 control OHCs) from 14 mice (8 DT-injected mice and 6 control mice). We studied the experimental mice 7 days after DT injection. This time delay was selected to give adequate time to ensure that feedback to the OHCs could happen and was based on our knowledge that IHCs were gone by 3 days after DT injection and the fact that 7 days after noise exposure, OHC prestin is upregulated (Xia et al. 2013).

Baseline currents measured immediately after achieving the whole cell configuration using a holding potential of −80 mV were similar between cohorts (DT-injected cohort: 0.06 ± 0.07 nA and control cohort: 0.2 ± 0.2 nA, P = 0.14 by t-test). We then measured membrane capacitance and found the standard bell-shaped curve characteristic of prestin function, with no obvious difference between DT-injected mice and control mice (Fig. 3A). To quantify prestin function, capacitance-versus-voltage curves were fit to the first derivative of a Boltzmann function. We then calculated linear capacitance, NLC, Qmax, charge density, α, and V1/2. None of these parameters were different between the two cohorts of mice (P > 0.1 for each comparison; Fig. 3B), indicating that both cohorts of mice had similar functional prestin levels. The lack of a difference in linear capacitance between the two groups indicates that the OHCs we studied were the same size, since this serves as a surrogate measure of cell surface area (Huang and Santos-Sacchi 1993).

Finally, we performed quantitative real-time PCR to determine whether prestin mRNA levels were affected in DT-injected mice. For each cohort, we wanted to normalize prestin mRNA to myosin VIIa mRNA, an internal hair cell reference gene (Duncan et al. 2006; Xia et al., 2010 and 2013). Since the direct effect of DT-induced loss of IHCs will be to reduce myosin VIIa levels without affecting prestin levels, this ratio would be expected to increase even if prestin is not upregulated. Therefore, we measured this impact by also normalizing prestin and myosin VIIa to GAPDH, a general housekeeping gene found in all cells. Furthermore, we calculated the predicted changes for these ratios using the cell counts shown in Fig. 2C (Xia et al. 2013). There were no significant differences in the prestin-to-GAPDH ratio, myosin VIIa-to-GAPDH ratio, or prestin-to-myosin VIIa ratio between DT-injected mice and predicted values (P > 0.15 for each comparison; Fig. 3C). In addition, there were no significant changes in these ratios in DT-injected mice compared with control mice (P > 0.08 for each comparison). Taken together, these results indicate that prestin transcription, prestin protein function, and the nonlinearities amplified by prestin in vivo that lead to DPOAEs are unaffected when hearing loss is caused by blocking afferent auditory input. Thus, the concept that centrally mediated feedback increases prestin levels in states of hearing loss was proven false. This indicates that local factors within the epithelium likely provide the feedback necessary to regulate OHC prestin levels.

Prestin levels in TectaC1509G mice. To address how local feedback could regulate prestin, we tested whether the direct effect of stimulating the stereociliary bundle of the OHC correlated with prestin levels using the TectaC1509G mutant mouse (Xia et al. 2010). In this mouse strain, the anatomy of the tectorial membrane varies with the genotype of the mouse.
Tecta\textsuperscript{+/+} mice have a tectorial membrane that is attached to the stereociliary bundle of every OHC within the epithelium, Tecta\textsuperscript{C1509G/+} mice have a tectorial membrane that is only connected to OHCs that sit within the first row (the row closest to the pillar cells), and Tecta\textsuperscript{C1509G/C1509G} mice have a tectorial membrane that is not attached to any OHCs. In heterozygous mice, the altered tectorial membrane anatomy, in which it has a functional connection to OHCs in the first row,
previous studies, based on whole organ physiology and whole cochlea mRNA analysis, suggested that there was more prestin in Tecta<sup>C1509G/+</sup> and Tecta<sup>C1509G/C1509G</sup> mice compared with Tecta<sup>+/+</sup> mice (Liu et al. 2011; Xia et al. 2010). However, no single cell analyses or row-specific functional assays for prestin levels have been done to test whether this represents functional prestin protein within the plasma membrane.

Here, we patch-clamped OHCs from adult mice at the age of P30 so that their prestin levels would be mature (Abe et al. 2007; Belyantseva et al. 2000; He et al. 1994). All OHCs studied were within the first row, so if OHC stimulation relates to prestin level, Tecta<sup>C1509G/+</sup> OHCs should have the same prestin level as Tecta<sup>+/+</sup> OHCs, whereas Tecta<sup>C1509G/C1509G</sup> OHCs should have an elevated prestin level. We recorded from nine OHCs from six Tecta<sup>C1509G/+</sup> mice, eight OHCs from five Tecta<sup>C1509G/+</sup> mice, and nine OHCs from six Tecta<sup>C1509G/C1509G</sup> mice. After the whole cell configuration had been achieved, average baseline currents using a holding potential of −80 mV were similar between the cohorts (Tecta<sup>C1509G/+</sup> mice: 0.2 ± 0.2 nA, Tecta<sup>C1509G/C1509G</sup> mice: 0.07 ± 0.08 nA, and Tecta<sup>C1509G/C1509G</sup> mice: 0.08 ± 0.2 nA, P = 0.3 by ANOVA).

We then measured membrane capacitance over the voltage range (Fig. 4). NLC, Q<sub>max</sub>, and charge density were significantly elevated in first-row OHCs from Tecta<sup>C1509G/C1509G</sup> mice compared with first-row OHCs from Tecta<sup>+/+</sup> mice (P < 0.007 for all comparisons by t-test). In contrast, first-row OHCs from Tecta<sup>C1509G/C1509G</sup> mice were no different than first-row OHCs from Tecta<sup>+/+</sup> mice (P > 0.7 for all comparisons by t-test). Linear capacitance, α, and V<sub>1/2</sub> values were not significantly different between the three genotypes (P > 0.2 for all comparisons by one-way ANOVA). These data indicate that OHCs within the first row that are not attached to the tectorial membrane have increased functional prestin. In addition, the lack of a difference in linear capacitance indicates that the OHC is able to upregulate functional prestin levels without a change in surface area.

To further test this concept, we proceeded to patch additional OHCs from the second and third rows in each genotype and compared all three rows among all three genotypes (Fig. 5A). From nine Tecta<sup>C1509G/+</sup> mice, we recorded from eight cells in the second row and seven cells in the third row. In 10 Tecta<sup>C1509G/+</sup> mice, we recorded from 8 cells in the second row and 10 cells in the third row. In eight Tecta<sup>C1509G/C1509G</sup> mice, we recorded from eight cells in the second row and eight cells in the third row. Average baseline currents at a holding potential of −80 mV were similar to previous data, and there were no differences between the three rows or three genotypes (P > 0.3 by two-way ANOVA). Consistent with the observations in first-row OHCs, membrane capacitance measures revealed increased functional prestin levels in OHCs that were not attached to the tectorial membrane (Fig. 5, B and C). This was evidenced by increased NLC, Q<sub>max</sub>, and charge density in OHCs from the second and third rows in Tecta<sup>C1509G/+</sup> mice and from all three rows in Tecta<sup>C1509G/C1509G</sup> mice (P < 0.001 for all comparisons by ANOVA followed by t-tests). In contrast, OHCs from all three rows in Tecta<sup>+/+</sup> mice had similar NLC, Q<sub>max</sub>, and charge density values (P > 0.6 by ANOVA). Linear capacitance and α values were not significantly different between the three genotypes or rows (P > 0.1 by two-way ANOVA). There was a slight difference in V<sub>1/2</sub> values between row OHCs in heterozygous mice (Liu et al. 2011).

Fig. 3. OHCs from DT-injected mice have normal levels of functional prestin. A: average capacitance as a function of voltage was measured in OHCs from control and DT-injected mice. B: linear capacitance, nonlinear capacitance, total charge moved (Q<sub>max</sub>), charge density, slope factor of voltage dependence (α), and voltage at peak capacitance (V<sub>1/2</sub>) were not significantly different between control and DT-injected mice. C: levels of prestin mRNA and myosin VIIa mRNA normalized to the level of GAPDH mRNA were similar between DT-injected mice and predicted values based on the cell counts (left and middle, respectively). In addition, the level of myosin VIIa mRNA was similar to the predicted value (right). None of the changes after DT injection were significantly different than control, P30, postnatal day 30.

but not the second or third rows, has been confirmed by multiple methods including histological sectioning, in situ visualization of the tectorial membrane free edge and the underlying OHC rows, in situ imaging of dynamic Ca<sup>2+</sup> changes within OHCs in each row during sound stimulation, in vivo cochlear microphonic recordings (Xia et al. 2010), and in situ optical imaging using optical coherence tomography (Gao et al. 2011). In addition, noise exposure produces loss of only first row OHCs in heterozygous mice (Liu et al. 2011). Our
the rows ($P = 0.02$ by two-way ANOVA), with a gradient toward a more negative $V_{1/2}$ value moving from the first row to the third row; however, there was no difference in $V_{1/2}$ values between the genotypes ($P = 0.07$ by two-way ANOVA). Taken together, these data confirm that OHCs attached to the tectorial membrane have normal prestin levels, whereas OHCs detached from the tectorial membrane have increased prestin levels. Thus, decreased stereociliary bundle stimulation correlates with increased prestin. The size of the OHCs, as assessed by linear capacitance, was unaffected.

Prestin upregulation occurs after the endocochlear potential develops. We acknowledge two possible mechanisms by which bundle stimulation might feedback onto prestin levels. One option is that there is a direct mechanical effect by which forces applied to the bundle in some way alter the mechanics of the cell and modulate prestin expression. Another option is that the transduction currents and receptor potential that develop within the soma of the OHC in response to the stimulation modulate prestin expression. We tested these two possibilities by measuring prestin levels during the period of postnatal cochlear development. The tectorial membrane malformation in $Tecta^{C1509G/C1509G}$ mice is present by $P5$ (Xia et al. 2010), prestin expression begins at $P4$–$P5$, prestin mRNA expression is maximal by $P10$ (Abe et al. 2007), and the stiffness of the OHC correlates strongly with the onset of prestin expression (Jensen-Smith and Hallworth 2007). Therefore, if mechanical forces stemming from tectorial membrane-induced deflections of the OHC stereocilia regulate prestin, differences in prestin expression should be detectable before $P10$. In contrast, if transduction currents and/or receptor potentials regulate prestin, the effect should not be seen until later since the endocochlear potential begins to rise at $P10$ and reaches adult levels at $\sim P15$ (Schmidt and Fernandez 1963). Thus, we addressed this question by measuring the developmental time course of prestin expression levels in $Tecta^{C1509G}$ mice.

We patch clamped first-row OHCs in all genotypes at $P10$, $P15$, and $P20$ (Fig. 6, A and B). Linear capacitance and $\alpha$ values were not significantly different between the three genotypes at any age ($P > 0.15$ for each measure by two-way

Fig. 4. OHCs in row 1 in $Tecta^{C1509G/C1509G}$ mice have elevated functional prestin levels compared with those in $Tecta^{+/+}$ and $Tecta^{C1509G/+}$ mice. A: average capacitance as a function of voltage in row 1 of OHCs in the three $Tecta$ genotypes. B: linear capacitance was not significantly different among all three genotypes, whereas nonlinear capacitance, $Q_{\text{max}}$, and charge density were higher in $Tecta^{C1509G/C1509G}$ mice compared with $Tecta^{+/+}$ and $Tecta^{C1509G/+}$ mice. $\alpha$ and $V_{1/2}$ were not significantly different between the groups. *$P < 0.05$ compared with $Tecta^{+/+}$ mice (by unpaired $t$-test).
ANOVA), but NLC, $V_{1/2}$, $Q_{\text{max}}$, and charge density demonstrated both age and genotype effects ($P < 0.01$ for each measure by two-way ANOVA). Further analyses revealed that NLC, $Q_{\text{max}}$, and charge density were all lower in Tecta$^{C1509G/C1509}$ mice compared with Tecta$^{+/+}$ and Tecta$^{C1509G/C1509}$ mice at P10 (one-way ANOVA followed by t-tests for each measure). However, at every older age (P15, P20, and P30), these values were higher (one-way ANOVA followed by t-tests for each measure). $V_{1/2}$ demonstrated age-related changes in Tecta$^{+/+}$ mice ($P < 0.01$ by one-way ANOVA) but not in the other two genotypes ($P > 0.2$ by one-way ANOVA). This suggests that there may be an activity-dependent component to development that is slowed in the mutants because of altered stimulation mechanics.

Quantitative real-time PCR was also performed to measure the prestin-to-myosin VIIa ratio (Fig. 6C). Prestin gene expres-
tion was higher in *Tecta*<sup>C1509G/C1509G</sup> mice compared with *Tecta*<sup>+/+</sup> mice at P15 (*P* = 0.01 by one-way ANOVA followed by *t*-tests) and higher in both *Tecta*<sup>C1509G/C1509G</sup> and *Tecta*<sup>C1509G+/+</sup> mice at P30 (*P* = 0.002 and *P* = 0.034, respectively). Taken together, these data indicate that functional prestin expression increases in OHCs not attached to the tectorial membrane only after P10 and, thus, after the endocochlear potential develops.

**DISCUSSION**

Given the central role of prestin protein in producing OHC electromotility, it is reasonable to assume that regulating the amount of prestin within the OHC is important to normal hearing. In the present study, we show that functional prestin protein can be increased above typically measured levels and that this process does not involve centrally mediated efferent feedback. Furthermore, we show that prestin regulation is stimulation dependent, in that OHCs stimulated by the tectorial membrane have a functional prestin level that is ~20% lower than those not stimulated by the tectorial membrane. This regulatory feedback mechanism depends on the cochlea being matured to a point where it has developed an endocochlear potential. Thus, while we cannot rule out a role for signaling between OHCs via reciprocal synapses in type II nerve fibers, our data are consistent with the concept that prestin expression within the OHC is locally regulated by the steady-state transducer bias current. Blocking the bias current [as occurs when the tectorial membrane is detached (Xia et al. 2010; Yuan et al. 2010)] or not having a bias current [as occurs before the development of the endocochlear potential] increases prestin expression. Since both prestin protein and mRNA are potentiated, the regulatory feedback mechanism appears to occur at the level of transcription.

The concept that centrally mediated feedback does not regulate prestin is consistent with previously published data demonstrating that *α9<sup>−/−</sup>* mice, in which the receptor that plays a dominant role in the efferent olivocochlear signal transmission (Eybalin 1993; Guinan Jr. and Guinan 2006), have normal auditory thresholds and OHC electromotility (Elgoyhen et al. 1994; He et al. 2004; Vetter et al. 1999). However, other efferent neurotransmitters have been identified in the efferent fibers (including GABA, CGRP, and various opioids) whose function remains unknown (Altschuler et al., 1984; Vetter et al. 1991; Fox and Altschuler, 1981 and 1984;
Maison et al. 2003; Wersinger and Fuchs 2011). Thus, the lack of an obvious difference in OHC function in e9−/− mice does not completely rule out a role for efferent feedback in regulating prestin levels in response to hearing loss regulated by higher brain centers. Unfortunately, the functional role of the efferent system in the sense of hearing remains poorly understood. However, our finding that severe hearing loss detected by higher brain centers does not lead to changes in OHC function, as measured by prestin levels, NLC, and DPOAE thresholds, is useful in that it suggests that the efferent system has little to no effect in a state of hearing loss.

In support of this idea, ototerlin−/− mice have no afferent response detectable by ABRs but have normal DPOAEs (Roux et al. 2006), which argues that OHC electromotility is unchanged in the presence of deafness and presumably a functioning efferent pathway. Similarly, centrally mediated feedback via thyroid hormone (TH) may be a critical regulator of prestin mRNA production and protein function since absence of TH causes deafness and significantly reduces prestin mRNA (Weber et al. 2002). However, electromotility is unaffected in OHCs deprived of TH, indicating that hearing impairment seen in TH-deficient models may actually be due to morphological or physiological changes in the cochlea rather than a problem regulating prestin (He et al. 2003).

The upregulation of prestin expression shown in previous studies have been in response to transient changes, such as noise exposure or salicylate injection. In Tecta−/− mice, however, the reduced stimulation of OHCs is inherited. How do they know to upregulate prestin? The simplest explanation of mechanism of how mechanical stimulation regulates prestin transcription is that regulation occurs individually within each OHC. Reduced steady-state bias currents from lack of tectorial membrane attachment would hyperpolarize the OHC in vivo, which may increase in prestin levels. There are many examples of activity-dependent protein synthesis, for example, this type of process is the underlying basis for some forms of long-term potentiation (for reviews, see Heise et al. 2014 and Schuman et al. 2006). In hair cells, this concept has been noted in organotypic cultures whereby OHC prestin levels decrease in response to depolarizing the membrane potential by increasing K+ concentration in the external solution (Mazurek et al. 2011). While the transcription factors Gata-3 and Carf are also modulated by this manipulation, further work is needed to identify the signal transduction pathway whereby transmembrane potential changes modulate prestin transcription. One additional feedback mechanism that should not be overlooked is the possibility of regional signaling. Type II nerve fibers innervate multiple OHCs along a single row, often with reciprocal synapses (Francis and Nadol Jr. 1993; Spoendlin 1969). Thus, bidirectional communication could create a regional feedback mechanism that might play a role in regulating OHC function and prestin levels. Unfortunately, we cannot test for this possibility as we are not aware of an animal model where type II neurons can be eliminated.

Finally, the reason why the DT injection preferentially kills IHCs in adult CBA mice is unclear. Infection by Corynebacterium diphtheriae, the bacterium that naturally produces DT, is a known cause of sensorineural hearing loss, but the pathophysiology is not known (Schubert et al. 2001). Mouse cells are generally highly resistant to DT and can survive 4,000 times the DT dose required to kill a human cell (Pappenheimer et al. 1982). Interestingly, among mouse cells, neural cells are 200 times more sensitive to DT than other somatic cells (Altt and Cavanagh 1969; Pappenheimer et al. 1982). In addition, the mRNA that codes for the protein that DT damages in humans, eukaryotic elongation factor 2 (Honjo et al. 1971), is expressed in a higher level in IHCs than in OHCs (606 vs. 432) (Liu et al. 2014; National Center for Biotechnology Information 2014). This may in part underlie the differential susceptibility of adult hair cells to DT. In addition, DT may have other effects that were undetected by the studies to assess cochlear function that we performed. While transgenic mouse models have been used in which adult utricular hair cells selectively express the DT receptor to produce hair cell death in studies of regeneration (Golub et al. 2012), we are unaware of any DT studies involving nonhair cell targets in adult mice where hearing has been assessed. To date, all other inner ear studies involving the administration of exogenous DT have used neonatal mice (Burns et al. 2012; Cox et al. 2014; Mellado Lagarde et al. 2013). Thus, as far as we are aware, this is the first report of this mouse model of IHC loss.

Why might a mechanism to modulate prestin levels based on stereociliary bundle stimulation have evolved? The primary function of OHCs is to produce mechanical force to amplify the vibration of the cochlear partition (Gao et al. 2014; Oghalai 2004b). Their stereociliary bundle is fixed to the undersurface of the tectorial membrane, unlike the stereociliary bundle of IHCs, which is untethered. To place the OHC stereociliary bundles at the bias point of maximum sensitivity, the center of the transduction curve, it would be beneficial for the length and/or stiffness of the OHC to be adjustable. Both of these factors are regulated by prestin. For example, the prestin−/− mouse has OHCs that are shorter and less stiff than OHCs from wild-type mice (Dallos et al. 2008; Liberman et al. 2002). Over time, aging, noise trauma, ototoxic exposure, or other factors often lead to progressive OHC loss, which may alter the static position of the stereociliary bundles of residual OHCs. This feedback might then upregulate prestin expression so as to return to the proper bias point. Similarly, during development, such a mechanism may help to increase prestin expression initially and then turn down prestin expression when the correct bias point is reached. Thus, an evolutionary mechanism for prestin regulation within individual OHCs may have developed to maintain optimal auditory sensitivity in the face of slow changes over time.

Acknowledgments

Artwork was created by Chris Gralapp.

Grants

This work was funded by Department of Defense Grant W81XWH-11-2-0004, National Institute on Deafness and Other Communication Disorders Grants DC-014450, DC-013774, DC-003896, and DC-010363, the Howard Hughes Medical Institute, and the Stanford Society for Physician Scholars.

Disclosures

No conflicts of interest, financial or otherwise, are declared by the author(s).

Author Contributions

Author contributions: Y.S., A.X., H.Y.L., A.J.R., and J.S.O. conception and design of research; Y.S., A.X., H.Y.L., R.W., and J.S.O. performed experi-
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