Parallel Sites Implicate Functional Convergence of the Hearing Gene Prestin among Echolocating Mammals

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Abstract

Echolocation is a sensory system whereby certain mammals navigate and forage using sound waves, usually in environments where visibility is limited. Curiously, echolocation has evolved independently in bats and whales, which occupy entirely different environments. Based on this phenotypic convergence, recent studies identified several echolocation-related genes with parallel sites at the protein sequence level among different echolocating mammals, and among these, prestin seems the most promising. Although previous studies analyzed the evolutionary mechanism of prestin, the functional roles of the parallel sites in the evolution of mammalian echolocation are not clear. By functional assays, we show that a key parameter of prestin function, 1/α, is increased in all echolocating mammals and that the N7T parallel substitution accounted for this functional convergence. Moreover, another parameter, V_{1/2}, was shifted toward the depolarization direction in a toothed whale, the bottlenose dolphin (Tursiops truncatus) and a constant-frequency (CF) bat, the Stoliczka’s trident bat (Aselliscus stoliczkanus). The parallel site of I384T between toothed whales and CF bats was responsible for this functional convergence. Furthermore, the two parameters (1/α and V_{1/2}) were correlated with mammalian high-frequency hearing, suggesting that the convergent changes of the prestin function in echolocating mammals may play important roles in mammalian echolocation. To our knowledge, these present the functional patterns of echolocation-related genes in echolocating mammals for the first time and rigorously demonstrate adaptive parallel evolution at the protein sequence level, paving the way to insights into the molecular mechanism underlying mammalian echolocation.

Key words: bat, dolphin, echolocation, convergent evolution, NLC.

Introduction

Echolocation is a typical convergent phenotype, which evolved independently in both whales and bats. In recent case and genome-wide studies, several genes have been identified as candidate genes related to echolocation from observations of parallel sites at the protein sequence level among echolocating mammals (Li et al. 2010; Liu, Cotton, et al. 2010; Davies et al. 2011; Liu et al. 2011; Liu, Han, et al. 2012; Shen et al. 2012; Parker et al. 2013). Although these studies documented parallel sites in these genes, none specified whether these genes were subject to adaptive evolution for mammalian echolocation by meeting all four key criteria necessary for illustrating adaptive evolution: 1) Whether functional convergence occurred, 2) whether parallel sites were observed, 3) whether parallel sites were under a common selective pressure, and 4) whether parallel substitutions caused the parallel functional changes (Zhang 2006). To date, prestin satisfies criteria 2 and 3 (Li et al. 2010), whereas all other reported cases of parallel protein evolution among echolocating mammals satisfy only criterion 2. As such, it remains unclear whether the observed parallel amino acid substitutions at the sequence level of prestin underlie the phenotypic convergence.

The membrane motor protein prestin, encoded by prestin (also named SLC26A5), drives the electromotility of mammalian outer hair cells (OHCs) on the hearing organ of Corti (Zheng et al. 2000), which provides the physiological basis of high-frequency sensitivity and frequency selectivity in mammalian hearing (Brownell et al. 1985; Ashmore 1987; Santos-Sacchi 1991; Dallos and Fakler 2002). Tightly packed prestin motors in the plasma membrane of the OHC lead to length changes of the whole cell, which then invariably necessitates a mobile charged particle that acts as a voltage sensor driven by voltage changes. Fast voltage-dependent charge movement is a hallmark of prestin that has previously been experimentally assessed as a nonlinear capacitance (NLC). Since this NLC is linked to cell motility and can be easily assayed experimentally, it is often used to evaluate the function of prestin (Santos-Sacchi 1991; Dallos and Fakler 2002).

Phylogenetic analysis of prestin sequence data grouped together the echolocating bats, which have the ability to detect high-frequency echolocating calls (Li et al. 2010; Liu, Cotton, et al. 2010), even though these bats are not monophyletic in their species tree (Teeling et al. 2005). In addition to echolocating bats, toothed whales are capable of sophisticated echolocation. Intriguingly, both toothed whales and...
sequences of prestin due to parallel evolution, but, more interestingly, prestin also appears to be under strong divergent evolution among echolocating mammals (Li et al. 2008, 2010; Liu, Cotton, et al. 2010). Previous studies suggested that the echolocation-related hearing gene prestin evolved under complex evolutionary trajectories, which would be consistent with the observed echolocation-related phenotypes. For example, although both toothed whales and echolocating bats can emit high-frequency echolocation in contrast to nonecholocating baleen whales (suborder: Mysticeti) and Old World fruit bats (family: Pteropodidae), their echolocation calls show a substantial diversity in shape, duration, and amplitude. This difference may be a result of how echolocation evolved in distinctively different environments (Schnitzler et al. 2003; Jones 2005; Jones and Teeling 2006).

In this study, to test whether prestin underwent adaptive parallel evolution, we examined the functional involvement of prestin and the roles of the parallel sites among echolocating mammals for echolocation. We examined the NLC of prestin using patch-clamp technology and then inferred the characteristic parameters to evaluate its functional pattern in echolocating mammals. Our results indicated that the values of the functional parameters $1/\alpha$ and $V_{1/2}$ of prestin changed during convergence among echolocating mammals, and the parallel sites accounted for the functional convergence. These new findings regarding the functional patterns of the hearing gene prestin in echolocating mammals offer new insights into the marked diversity of mammalian echolocation at the molecular level as well as the underlying genetic mechanisms responsible for echolocation, which evolved independently in bats and toothed whales.

**Results**

**Functional Convergence of Prestin among Echolocating Mammals**

EGFP-tagged prestin proteins from all the tested species (see Materials and Methods for details) exhibited membrane targeting and were generally observed 24 h after transfection of the HEK293 cells (one example from the prestin expression in vitro of the short-nosed fruit bat is presented in fig. 1A). Cells with strong, unambiguous membrane fluorescence were selected for further electrophysiological recordings. Figure 1B illustrates the representative recordings of NLC measured from the prestin proteins of the Stoliczka’s trident bat, the Rickett’s big-footed bat, the short-nosed fruit bat, the fin whale, the cow, and the gerbil. Compared with the negative control, an empty pEGFP-N1 vector ($n = 5$), the prestin protein of all seven species displayed a robust bell-shaped dependence on membrane potential (fig. 1B).

The voltage-dependent NLC of mammalian prestin is well characterized by the first derivative of the Boltzmann function, which describes charge movement (Santos-Sacchi 1991). For the gerbil (an outgroup of both the echolocating and nonecholocating bats and whales), measurements were obtained from 15 prestin-GFP expressing cells, and the mean values and standard deviations (SDs) of the fitted NLC curve yielded values of $Q_{\text{max}}/C_{\text{lin}} = 6.23 \pm 4.91 \text{ fC/pF}$, $V_{1/2} = -59.47 \pm 9.77 \text{ mV}$, and $1/\alpha = 34.0 \pm 6.8 \text{ mV}^{-1}$ (fig. 1C and supplementary fig. S1, Supplementary Material online). Compared with that of the outgroup, the functional parameters of the prestin from the short-nosed fruit bat, the fin whale, and the cow showed no significant differences (supplementary fig. S1, Supplementary Material online). Conversely, the function of prestin among echolocating mammals changed greatly, especially with regard to the functional parameters $V_{1/2}$ and $1/\alpha$. Compared with that in nonecholocating mammals (i.e., the short-nosed fruit bat vs. the Stoliczka’s trident bat and the Rickett’s big-footed bat, and the bottlenose dolphin vs. the fin whale), the value of the functional parameter $1/\alpha$ in the echolocating mammals increased significantly (fig. 1C; $P < 0.01$, Student’s t-test), although there was no significant difference among the tested echolocating mammals.

As shown above, prestin proteins of echolocating mammals exhibited functional convergence on the functional parameter $1/\alpha$. We further observed that the functional parameter $V_{1/2}$ of the prestin proteins from the Stoliczka’s trident bat and bottlenose dolphin was significantly shifted in the depolarization direction compared with those of other echolocating mammals, with $V_{1/2}$ values of $-44$ and $-46.9 \text{ mV}$ (fig. 1C; $P < 0.01$, Student’s t-test). Aside from $1/\alpha$ and $V_{1/2}$, another functional parameter of charge density ($Q_{\text{max}}/C_{\text{lin}}$) is worth noting. We observed no significant difference between nonecholocating and echolocating mammals, aside for the Rickett’s big-footed bat, in which the $Q_{\text{max}}/C_{\text{lin}}$ value of the prestin protein was significantly higher than that of the other compared species ($Q_{\text{max}}/C_{\text{lin}} = 13.3 \pm 5.8 \text{ fC/pF}$; $P < 0.01$, Student’s t-test; supplementary fig. S1, Supplementary Material online).

Taken together, these results suggest that functional convergence of prestin proteins occurred in the two parameters $1/\alpha$ and $V_{1/2}$ among echolocating mammals, indicating that prestin satisfied not only the second and third requirements we outlined but also the first one.

**Identification of Convergent/Parallel Sites of Prestin among Echolocating Mammals**

We next investigated whether the convergent function of prestin results from convergent/parallel sites. Previous studies documented a number of convergent/parallel sites by comparing prestin protein sequences of echolocating mammals. However, the estimation accuracy for the convergent/parallel sites mainly depended on the representative species used in those studies (Li et al. 2010; Liu, Cotton, et al. 2010). Among these representative species, we found that the species sampled in previous studies as representatives of the Yangochiroptera suborder did not provide a reasonable basis of comparison because these species only covered the two families (Vespertilionidae and Miniopteridae) with the closest phylogenetic relationship (Teeling et al. 2005). To avoid repeating this bias, we opted to add sequence

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**Fig. 1.** Voltage-dependent membrane capacitance (NLC) of HEK293 cells transiently transfected with *prestin* orthologs of echolocating and nonecholocating mammals. (A) Confocal images showing the plasma membrane location of the prestin-GFP constructs. (B) Representative NLC traces of mammalian prestin and the negative control. Different colors indicate different species. (C) The parameters $V_{1/2}$ and $1/\alpha$ obtained from fitting with the Boltzmann function are shown. Cow (*Bos taurus*), $V_{1/2} = -49.5 \pm 6.7$ mV, $1/\alpha = 34.1 \pm 4.9$ mV; gerbil (*Meriones unguiculatus*), $V_{1/2} = -59.5 \pm 9.7$ mV, $1/\alpha = 34 \pm 6.8$ mV; short-nosed fruit bat (*Cynopterus sphinx*), $V_{1/2} = -56.4 \pm 12$ mV, $1/\alpha = 33.9 \pm 4.9$ mV; the Stoliczka’s...
information from the oldest family in the Yangochiroptera suborder—in this case, a species of the black-bearded tomb bat (Taphozous melanopogon). This addition should make our estimation for convergent/parallel sites more reliable because it is a member of the Emballonuridae family, the outmost family of the Yangochiroptera suborder (Teeling et al. 2005). Finally, we obtained the full-length coding sequences of prestin from 40 species, which included seven echolocating constant-frequency (CF) bats, nine echolocating FM bats, four nonecholocating Old World fruit bats, seven echolocating toothed whales, three nonecholocating baleen whales, and ten other nonecholocating mammals (fig. 2). Comparison of these prestin protein sequences showed that the amino acid at position 7 of prestin is a threonine (Thr or T) in all echolocating mammals but an asparagine (Asn or N) in all nonecholocating mammals, suggesting that this site experienced a parallel change on the ancestral branches of echolocating mammals (fig. 2), consistent with the results of the previous studies (Li et al. 2010; Liu, Cotton, et al. 2010; Liu, Rossiter, et al. 2010).

We also tried to identify the convergent/parallel sites between CF bats and toothed whales to investigate the underlying molecular mechanisms of their functional convergence in the parameter $V_{1/2}$. To identify the convergent/parallel sites, we inferred the prestin protein sequences for each interior node of the species tree using PAML4.0 (Yang 2007). A comparison of the ancestral and currently extant prestin sequences showed that four parallel substitutions, I384T, S392A, L566F, and N685S, occurred independently on the ancestral branches of CF bats and toothed whales (fig. 2). Statistical analysis showed that there were significantly more parallel sites than expected from random events on the ancestry of echolocating mammals but an asparagine (Asn or N) in all nonecholocating mammals (fig. 2). These results provide the first direct evidence that the parallel sites of N7T and I384T are responsible for the functional convergence of the 1/α value of prestin in echolocating mammals (fig. 3A). Although the $V_{1/2}$ values were not significantly influenced, the mutagenesis resulted in different effects on the functional parameter $Q_{\text{max}}/C_{\text{lin}}$ in the different genetic backbones (fig. 3).

The Parallel Change of I384T Accounts for Functional Convergence in the Parameter $V_{1/2}$ of Prestin between CF Bats and Toothed Whales

We next created single replacement mutations of I384T, S392A, L566F, and N685S in the prestiins of the short-nosed fruit bat and the fin whale and used a whole-cell patch-clamp to determine the impact on the prestin NLC function in these mutants. All the mutants displayed robust bell-shaped voltage-dependent NLC, and the single replacement of I384T resulted in shifted $V_{1/2}$ values to a more depolarized potential compared with their corresponding WT prestiins ($P < 0.05$, Student’s t-test; fig. 3B). The trend of change for the functional parameter $V_{1/2}$ is consistent with that from nonecholocating to echolocating mammals (fig. 1C). Unexpectedly though, this parallel site also significantly decreased the $Q_{\text{max}}/C_{\text{lin}}$ values ($P < 0.05$, $P < 0.01$, Student’s t-test; fig. 3C). The other parallel sites, S392A, L566F, and N685S, did not affect the convergent parameter $V_{1/2}$ between CF bats and toothed whales, but they substantially increased the parameter 1/α (fig. 3D). Rather intriguingly, the parallel sites of L566F and N685S increased the values of $Q_{\text{max}}/C_{\text{lin}}$ but I384T decreased them. The mutagenesis of S392A surprisingly displayed a different influence on the functional parameter $Q_{\text{max}}/C_{\text{lin}}$ of the NLC in the nonecholocating mammals (fin whale and short-nosed fruit bat).

These results provide the first direct evidence that the parallel sites of N7T and I384T are responsible for the functional convergence in the parameters 1/α and $V_{1/2}$ among echolocating mammals, indicating that the hearing gene of prestin satisfies all four requirements for adaptive parallel evolution, meaning that it plays an important role in mammalian echolocation.

Discussion

According to the four requirements for adaptive parallel evolution (Zhang 2002), the previous conclusions regarding prestin were not sufficiently rigorous to verify that the hearing genes of prestin underwent adaptive parallel evolution due to the absence of functional investigation. In this study, we accordingly sought to characterize the function of the hearing gene prestin in echolocating and nonecholocating mammals.

Our comparison of echolocating mammals with their nonecholocating cohorts as well as an outgroup showed a functional convergence in the parameter 1/α of prestin NLC among echolocating mammals and in the parameter $V_{1/2}$ among nonecholocating mammals.

The Parallel Site of N7T Is Essential for Enhanced 1/α Values of Prestin among Echolocating Mammals

We first replaced the seventh amino acid, Asn, in the prestin of nonecholocating mammals (the fin whale and the short-nosed fruit bat) with the amino acid Thr from the prestin of echolocating mammals to determine its contribution to the functional convergence for the parameter 1/α. The 1/α values of the mutants increased significantly compared with the corresponding wild types (WTs), suggesting that the parallel change of Asn to Thr accounts for the functional convergence of the 1/α value of prestin in echolocating mammals (fig. 3A). Although the $V_{1/2}$ values were not significantly influenced, the mutagenesis resulted in different effects on the functional parameter $Q_{\text{max}}/C_{\text{lin}}$ in the different genetic backbones (fig. 3).
of NLC between the Stoliczka’s trident bat and the bottlenose dolphin. In attempting to discern the roles that parallel sites played in the convergent functional parameters of NLC, we mutated these residues individually and found that the parallel sites N7T and I384T caused the functional convergence. Taken together with evolutionary analyses, these findings offered the first substantial evidence to support the notion that the hearing gene of prestin underwent adaptive parallel evolution among echolocating mammals.

Although the evidence supporting prestin’s adaptive parallel evolution is exciting, it leads to a more fundamental question—why do these parallel sites influence the function of prestin so intensely? Although the three-dimensional structure of prestin has not been analyzed, secondary topology models have been documented using functional and modeling assays (Zheng et al. 2001; Deak et al. 2005; Navaratnam et al. 2005; Mio et al. 2008). In these models, the positions of the parallel sites were the same. For example, position 7 is in

**Fig. 2.** Parallel sites among echolocating mammals mapped onto the mammalian species tree. Red lineages indicate echolocating mammals. Species names with green boxes indicate that the prestin function of these species have been examined in this study. Blue amino acid substitutions are parallel with all groups of echolocating mammals. Black amino acid substitutions are parallel between CF bats and toothed whales.
**Fig. 3.** Parallel sites between echolocating mammals playing different roles for functional changes of prestin. Point mutations for parallel sites are based on the prestin background of two nonecholocating mammals, the short-nosed fruit bat (*Cynopterus sphinx*) and the fin whale (*Balaenoptera physalus*). (A) Mutating the parallel site N7T among echolocating mammals significantly increases the $1/\alpha$ value (for the *C. sphinx* mutant, $1/\alpha = 59.8 \pm 11.9$ mV, and for the *B. physalus* mutant, $1/\alpha = 71.1 \pm 13.1$ mV). The mutations have different influences on the functional parameter $Q_{\text{max}}/C_{\text{in}}$ (for the *C. sphinx* mutant, $Q_{\text{max}}/C_{\text{in}} = 12.3 \pm 8.4$ fC/pF, and for the *B. physalus* mutant, $Q_{\text{max}}/C_{\text{in}} = 3.5 \pm 1.4$ fC/pF). (B) Each of the parallel sites between the CF bats and
the amino terminus, which mediates homomultimerization of prestin (Navaratnam et al. 2005), the positions 384 and 392 fall in the transmembrane region, which is critical for anion transport (Bai et al. 2009; McGuire et al. 2010), and the positions 566 and 685 are located in the carboxyl terminus responsible for targeting to the cell membrane (Zheng et al. 2005). These similarities suggest that it is quite likely that these parallel sites play important roles in the function of prestin, which in turn is crucial for the high-frequency acoustic sensitivities possessed by echolocating mammals.

To explore the relationship between the functional parameters of prestin NLC and high-frequency hearing further, we collected data on the frequency of the best hearing sensitivity of the nonecholocating mammals (gerbil and cow) through audiograms (Ryan 1976; Hefner and Hefner 1983) as well as of the echolocating bats and whales obtained from a previous study (Liu, Rossiter, et al. 2010). We analyzed the relationships among these hearing frequencies and the functional parameters 1/α and V_{1/2} of prestin NLC among the species examined in the present study. As shown in figure 4, the values of 1/α were associated positively with the best hearing frequencies (R = 0.79; P = 0.037), which was supported to a certain degree by the decrease of 1/α in cells from the more basal cochlea, which is sensitive to high-frequency sound (Santos-Sacchi et al. 1998; Ashmore 2008), suggesting that attenuation for elementary charges moving across the membrane may favor mammalian echolocation. Additionally, the V_{1/2} values were negatively associated with the best hearing frequencies (R = −0.85; P = 0.015). After correcting for phylogeny using an independent contrasts test (Midford et al. 2005; Maddison WP and Maddison DR 2010), there was still a significant correlation between the V_{1/2} values and the best hearing frequencies (R = −0.82; P = 0.046). The depolarizing shift in the voltage-dependent nonlinear peak capacitance in echolocating mammals can affect the anion-binding capability of prestin, with the consequence that the kinetics of prestin activation change, resulting in high-frequency hearing (Ashmore 2008). Although interesting, this result should be treated with caution because our data set contained functional data on the prestin from only six placental mammals. Some other nonmammalian animals and nonplacental mammals possess lower best hearing frequencies, but the peak of their prestin NLC did not significantly shift in the direction of negative potential (Tan et al. 2011; Liu, Li, et al. 2012; Tang et al. 2013). Accordingly, to make any definitive conclusions, more detailed and direct evidence is required to determine the relationship between the phenotypic effects of echolocation and hearing sensitivity and the functional parameters of prestin NLC.

During this study, we also observed that some parallel sites (except for N7T and I384T) led to the functional divergence in prestin among echolocating mammals (fig. 3). One likely explanation for this divergence lies in the epistatic interactions between these parallel sites because epistatic effects are an important, widespread genetic mechanism for molecular functions (Azevedo et al. 2006), and the combination of different sites can cause substantial changes in prestin function (Schaechinger et al. 2011). To verify this possibility, we generated the tetrad-mutant containing I384T, S392A, L566F, and N685S in the prestin background of the short-nosed fruit bat. The parameters 1/α and V_{1/2} of the tetrad mutant were substantially shifted in the direction of echolocating mammals (fig. 5), indicating that a combination of these parallel sites likely influences the function of prestin and that the interactions between parallel sites should not be overlooked when interpreting or attempting to elucidate the potential molecular mechanisms of convergent phenotypes. Aside from the parallel sites capable of causing differential functions of genes as described in a previous study (Zhang 2003), the divergent sites may also cause the same functional changes underlying phenotypic convergence (Rosenblum et al. 2010). Ultimately, then, the molecular mechanisms of convergent phenotypes are much more complicated than originally thought, and these results clearly call for a deeper exploration of the genetic basis of mammalian echolocation.

Materials and Methods

Taxonomic Coverage

To investigate the functional pattern of prestin in echolocating mammals, we selected three echolocating mammals for study: A Stoliczka’s trident bat (Aselliscus stolicczkanus), a Rickett’s big-footed bat (Myotis ricketti), and a bottlenose dolphin (Tursiops truncatus). These three serve as representatives, being derived from three different lineages known to possess echolocation. The Stoliczka’s trident bat and the Rickett’s big-footed bat belong to the family Hipposideridae in the suborder Yinpterochiroptera and the family Vesperilionidae in the suborder Yangochiroptera. Each has different echolocation calls; for example, the former can emit CF echolocation calls (and are termed CF bats), and the latter produces brief broadband signals (and are termed FM bats) (Jones and Teeling 2006). To compare the functional characteristics of prestin between these echolocating samples and nonecholocating mammals, we chose the cohorts of a nonecholocating bat (the short-nosed fruit bat, Cynopterus sphinx) and a nonecholocating whale (the fin whale, Balaenoptera physalus) as well as the cow (Bos taurus). We also selected the gerbil (Meriones unguiculatus) as an outgroup.

PCR Amplification and Sequencing

We obtained the prestin coding region of the black-bearded tomb bat (T. melanopogon), which is from the outermost family of Emballonuridae in the Yangochiroptera suborder. We used RT–PCR to amplify prestin from total RNA isolated from brain tissues and cochlear organs. For the first-strand
Identification of Convergent/Parallel Sites among Echolocating Mammals

The parallel/convergent sites among echolocating mammals were identified according to the methods previously described (Liu et al. 2011). In detail, we inferred ancestral prestin protein sequences for each interior node of the 40 species-tree using maximum-likelihood and MP methods of PAAML4.0 (Yang 2007). We used the species tree as a basis to construct ancestral sequences because this tree provides a more reliable evolutionary trajectory than the gene tree. Ancestral inferences appeared reliable because mean posterior probabilities for the entire protein exceeded 99% for all the nodes. We next looked for either parallel or convergent changes by comparing ancestral and extant prestin protein sequences. Although parallel changes are required to have the same descendant amino acid from the same ancestral amino acid, convergent evolution occurs from a different ancestral amino acid. We calculated the probability that the observed number of parallel or convergent sites exceeded that expected by random chance using a statistical method (Zhang and Kumar 1997).

Gene Synthesis, Cell Culture, and Transient Transfection

We chose seven mammalian species to serve as representative samples in our examination of prestin function, including two echolocating bats (A. stoliczkanus and M. ricketti), one non-echolocating bat (C. sphinx), one toothed whale (T. truncatus), one non-echolocating whale (B. physalus), and the cow (B. taurus) as well as the gerbil (M. unguiculatus) to act as an outgroup. The entire coding regions of prestin from these species were synthesized (Generay, CN) and cloned into the expression vector pEGFP-N1 (Clontech), yielding C-terminal GFP fusion constructs. The correct orientation and reading frame were verified by sequencing analysis. The expression vector for the gerbil’s prestin was a gift from Prof. Dallos’ laboratory at Northwestern University (USA).

The HEK293 cells were grown in 35-mm dishes containing Dulbecco’s modified Eagle’s medium supplemented with 10% bovine calf serum (Invitrogen). When the cell confluence reached roughly 50–60% of the surface area of the dishes, transfection of the prestin and pEGFP-N1 fusion constructs (3 μg) were accomplished using Lipofectamine 2000 transfection reagent (10 μl; Invitrogen). After 24–48 h incubation, the successfully transfected cells were used for NLC measurements.

Electrophysiological Experiments for NLC Measurements

NLC was measured using whole-cell patch-clamp recordings performed with a HEKA EPC 10 USB amplifier (HEKA Instruments Inc.) controlled by Patchmaster software (HEKA Instruments Inc.) at room temperature (22–26 °C). Electrodes were pulled from borosilicate glass with resistances of 2.5–4 MΩ and filled with the internal solution containing 140 mM CsCl, 2 mM MgCl2, 10 mM EGTA, and 10 mM HEPES. The cells were bathed during the recordings in an

cDNA synthesis, 1 μg of total RNA was reverse transcribed in a volume of 20 μl. Primers used in this study were previously described (Li et al. 2008). All products were isolated from a 1.5% agarose gel and cloned into the T-vector. Positive clones were sequenced in both directions using Big Dye Terminator on an ABI 3730 Sequencer. The sequence was deposited into GenBank (accession number: KF758787). Other mammalian prestin sequences used in this study were obtained from previously published reports (Li et al. 2008, 2010; Liu, Cotton, et al. 2010; Liu, Rossiter, et al. 2010).
external solution containing 120 mM NaCl, 20 mM TEA-Cl, 2 mM CoCl₂, 2 mM MgCl₂, 10 mM HEPES, and 5 mM glucose. Both solutions were adjusted to pH 7.2. Osomolarities of the internal and external solutions were adjusted to 300 and 320 mOsm/L, respectively, with glucose. Whole-cell membrane capacitance (Cₘ) was measured using the sine + DC software lock-in function of Patchmaster to obtain the parameters of charge movement. Voltage-dependent NLC was assessed by recording Cₘ during voltage ramps with a slope of 0.16 V/s, as described previously (Oliver and Fakler 1999; Schaechinger et al. 2011), and plotted as a function of membrane voltage (Vₘ). NLC was quantified by fitting with the derivative of a two-state Boltzmann function:

\[
C_m = \frac{Q_{\text{max}}}{\exp{[\alpha(V_m - V_{1/2})](1 + \exp{[-\alpha(V_m - V_{1/2})])}} + C_\text{lin},
\]

where \(Q_{\text{max}}\) is the maximum charge transfer, \(V_{1/2}\) is the voltage at which the maximum charge is equally distributed across the membrane, \(C_\text{lin}\) is the linear capacitance, and \(\alpha\) is the slope factor of the voltage dependence of the charge transfer. \(C_\text{lin}\) is proportional to the surface area of the membrane (cell size). To compare the magnitude of NLC obtained from different cells with different levels of prestin expression as a function of cell size, we normalized the NLC by the linear capacitance of the cells. Because differences in \(Q_{\text{max}}\) could have been caused by cell size, the charge movement was normalized to \(C_\text{lin}\). This quantity, designated as charge density, had units of femtocoulomb per picofarad.

Confocal Imaging

Membrane-associated expression of WT and mutant prestin was verified 24 h after transfection by confocal imaging. We first used 4% paraformaldehyde to fix HEK293 cells transfected with the expression vectors and stained the cell nuclei using 4′,6-diamidino-2-phenylindole. Confocal imaging was performed with an Olympus IX 81 microscope using a 100× oil immersion objective.

Supplementary Material

Supplementary figure S1 is available at Molecular Biology and Evolution online (http://www.mbe.oxfordjournals.org/).

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