Published in final edited form as:

J Neurosci Res. 2011 November ; 89(11): 1747–1760. doi:10.1002/jnr.22713.

Identification and Quantification of Full-Length BK Channel Variants in the Developing Mouse Cochlea

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Abstract

Maxi K⁺ (BK) channel diversity is attributed to alternative splicing in the *kcnma1* gene. The resultant variants manifest themselves in different cell types, tissues and functions such as excitation, metabolism, and signaling. Immuno-electron microscopy revealed immunogold particle labeling of BK in apical and basal regions of inner and outer hair cells, respectively. Additional labeling occurs in Deiters' cells and the inner mitochondrial membrane. Identification of full-length sequences reveals 27 BK variants from embryonic and postnatal mouse inner ear, per classification by tail motif, VYR, DEC, and ERL, and by exon usage. Three predicted start codons are found encoding MAN, MSS, and MDA, of which MDA shows the greatest expression through all stages in development, whereas MAN is undetectable. Complex splice sites occur between exons 9 and 10, and 21 and 23. Spliced-in/out exons between 8 and 10 reveal a short fragment composed of exons 8+10, detectable on postnatal-day (PD) 14 and PD30, and a longer fragment composed of exons 8+9+10 that upregulates on embryonic-day (ED) 14. Spliced-in exons 22 or 23 are expressed on ED14 but decrease over time; however, exon 22 increases again on PD34. Using tail specific primers, qRT-PCR from ED14, PD4, 14, and 30, show that BK-VYR and -ERL dominate expression on ED14, whereas DEC dominates after birth in all cochlear regions. The localization of BK and the changes in expression of its exons and tail types, by alternative splicing during development, may contribute to cochlear organization, acquisition of hearing, and intracellular signaling.

Keywords

cochlea; BK channel; splice variants; embryonic development; hair cell; immuno-electron microscopy

Introduction

Among the mediators of signal processing in the cochlea is the large conductance Ca²⁺-activated K⁺ or BK channel, found in hair cells and spiral ganglion neurons. The BK α -subunit is a pore-forming membrane protein composed of tetramers that can pair with modulatory β -subunits. The α -subunit consists of core (S0-S8) and tail (S9 and S10) domains. The core contains seven putative transmembrane segments (S0–S6), which includes a K⁺-selective pore region between S5 and S6. S0 consists of a transmembrane region and an extracellular N-terminus that couples with β -subunits. BK contains a long cytoplasmic C-terminus that comprises approximately 70% of the protein. This region contains four hydrophobic regions (S7-S10), with S7 and S8 included in the regulator of

conductance of K^+ 1 (RCK1) domain that encompass Ca^{2+} sensors. A potential RCK2 domain encompasses S9 and S10, and five consecutive aspartic acid residues of the Ca^{2+} bowl, which contains high-affinity Ca^{2+} binding sites (Cui et al., 2009 for review).

BK is an α -helical transmembrane protein located not only in the plasmalemma but also in the inner mitochondrial membrane and possibly the nuclear envelope (Yamashita et al., 2006; O'Rourke, 2007). The C-tail of BK is represented by three phenotypes: VYR, DEC, and ERL, which can regulate channel distribution to different cellular compartments (Ma et al., 2007). VYR and ERL types translocate to the cell surface at two or more times the rate of DEC (Kim et al., 2007a; Kim et al., 2010). DEC binds to proteins, such as nephrin, which does not interact with BK-VYR and -ERL (Kim et al., 2008), and to the PDZ domains of scaffolding proteins that regulate BK-DEC trafficking (Ridgway et al., 2009). Thus, C-terminal tail motifs are important for localization.

The *Kcnma1* gene, located on chromosome 14, encodes the mouse BK channel and has a genomic sequence of 705445 base pairs (Acc. No. NC_000080), while the spliced *Kcnma1* transcript with 29 exons (Acc. No. NM_010610) incorporates exons described herein. Alternative splicing generates diversity between species and tissues (Fodor et al., 2009). Exon 1 of the N-terminus has three predicted initiation codons in the same frame. A cluster of glycine or serine residues located between the first and second, or second and third methionines, respectively, characterizes this extracellular region. Exon 22, known as the STress axis-Regulated EXon (STREX), is located between RCK1 and 2. STREX expression can vary with age, showing high expression in embryonic brain (MacDonald et al., 2006) and a decrease, loss (ZERO), or replacement by other exons (Naruse et al., 2009) in adults. Thus, alternative splicing in this region is unique and variable.

The structural and functional maturation of inner and outer hair cells on the Organ of Corti during postnatal days (PD) 5-13 in the mouse reflects the onset of hearing. Patch clamp recordings show that the mouse acquires functional BK currents postnatally, beginning with a current activated by Ca^{2+} from intracellular stores (PD10-12), followed by a current activated by extracellular Ca^{2+} entering the cell via L-type Ca^{2+} channels (PD 12-14) (Kros, 2007 for review).

In the present study, BK diversity is examined through its localization in the sensory epithelium using immuno-electron microscopy and through cloned full-length variants and comparisons against GenBank (NCBI) data using BLAST. We report BK expression in sensory/neural and supporting cells, including organelles, and the cloning of 27 variants that are different from each other and from full-length cDNAs of mouse BK reported in GenBank. Variant expression differs at different developmental stages, which provide clues for conditional splicing-in/out of exons through semi- and quantified analyses of expression. In addition, we show that C-terminus tail variants, VYR, DEC, and ERL vary in expression during development as well as along the tonotopic axis.

Materials and Methods

Immuno-Electron Microscopy

Tympanic bullae were removed rapidly from 30 day-old CBA/J mice and fixed by immersion through the round window and a small hole created in the apex with 4% paraformaldehyde/0.1% glutaraldehyde (Ted Pella, Redding, CA) in 0.1M phosphate buffer pH 7.4 for 2 h at room temperature (RT). Tissues were then rinsed in PBS followed by partial or complete removal of the bony walls of the cochlea and decalcification for 3 days at RT in 5 mM EDTA/0.1 M phosphate buffer. Tissues were washed over the course of 2 h at 15 min intervals and then dehydrated as follows: 1 × 15 min in 50% EtOH, 2 × 30 min in

70% EtOH and 1 × 30 min in 85% EtOH. Infiltration with LR White medium grade resin (Ted Pella) involved placing tissues for 1 h in 2:1 resin:70% EtOH, followed by 2 × 1 h in 100% resin and overnight. The next day, tissues were placed in 100% resin for 1 h, embedded in 00 gelatin capsules and cured at 50°C for 48 h. Sections of gold-interference thickness were collected on Gilder 200 mesh nickel grids using a Sorvall Porter-Blum ultramicrotome and dried overnight.

Immunostaining was done using the post-embedding technique. Sections were rehydrated 3 × 5 min in TBS pH 7.4 and free aldehyde groups were blocked for 30 min in 50 mM glycine/TBS. Blocking solution consisted of 5% normal goat serum/1% BSA/0.1% cold-water fish gelatin/1% Tween 20/TBS. After 30 min, sections were rinsed briefly in 0.1% BSA-c (Ted Pella) followed by overnight (O/N) incubation at RT in anti-BK polyclonal antibody (1:25, Abcam, Cambridge, MA; epitope human aa 945-961) diluted in 0.2% BSA-c/1% Tween 20/TBS. The next day, sections were washed 3 × 5 min in TBS pH 8.0 and incubated in goat anti-rabbit IgG conjugated with 10 nm Au particles (BBL, Ted Pella) diluted 1:10 in 0.1% BSA-c/1% Tween 20/TBS pH 8.0 for 90 min. After several washes in TBS followed by water, sections were osmicated (2%) for 15 min, rinsed and then stained with 1% KMnO₄ (aq) for 30 s, rinsed, placed in 0.025% citric acid for 45 s, rinsed again and then stained with 2% aqueous uranyl acetate for 30 min. Sections were permitted to dry O/N. Tissues were imaged using an FEI Morgagni Model 268D TEM at 60 kV.

Tissue Preparation and Total RNA Extraction

Two- and four-week-old male and pregnant female CBA/J mice (Harlan) were used for these experiments under procedures approved by the Institutional Animal Care and Use Committee of the University of South Florida College of Medicine and conform to the guidelines established by the NIH. Mice were anesthetized with Isoflurane and euthanized by decapitation (Butler Animal Health Supply, Dublin, OH). The head was bisected and tissues removed as described for each specific procedure below. Tissues were rinsed in diethylpyrocarbonate (DEPC)-treated saline and stored at -80°C. In addition, sensory epithelia dissected from PD30 mice were divided into apical, middle, and basal turns for studies of different regions. Tissues were sonicated at settings 1-2 for 10 s using a Sonic Dismembrator Model 100 (Fisher Scientific, Pittsburgh, PA) and total RNA extracted using an RNeasy kit (Qiagen, Valencia, CA). Total RNA from each age group was stored at -80°C.

Cloning of Full-length Mouse BK Variants

VYR, DEC, and ERL type variants from the mouse BK α -subunit were cloned using total RNA extracted from PD14 and PD30 mouse whole cochleae by RT-PCR. VYR and DEC types from PD30 mouse cochlea were cloned with a BglII site at the 5' end of the forward primer, 5'-GGAAGATCTCCCAAGATGGATGCGCTCATCA-3' and a SalI site at the 5' end of the reverse primer, 5'-ACGCGTCGACAGTTCTGGTCTCCTGGG AGT-3'. Thermal cycling was performed for each cloning using a PTC-200 DNA Engine (MJ Research, Boston, MA) under the following conditions: 1 cycle at 50°C for 30 min, 94°C for 2 min, 35 cycles at 94°C for 15 s, 55°C for 30 s, 68°C for 4 min, followed by 1 cycle at 72°C for 10 min. PCR products were purified using a QIAquick PCR Purification Kit (Qiagen) and cut with BglII and SalI at 37°C overnight. Inserts were subjected to gel-electrophoresis, purified with a StrataPrep DNA Gel Extraction Kit (Stratagene, La Jolla, CA), and then ligated complementarily into the BamHI (5') and XhoI (3') sites of pcDNA3.1(+) plasmid (Invitrogen). The ERL type was TA-cloned into pcDNA3.1/NT-GFP-TOPO® plasmid (Invitrogen) using a SuperScript™ One-Step RT-PCR for Long Templates kit (Invitrogen), using the forward primer, 5'-CGCGGATCCCCCAA GATGGATGCGCTCATCATA CCGGTGA-3' and the reverse primer, 5'-TCAAAGCCGCTCTTCTGAACG T-3'.

The hair cells of the cochlea express at least two functional BK channel types by PD14, as defined by current recordings (Kros, 2007). Thus, total RNA from PD14 whole cochleae was obtained to clone variants of the three tail types using RT-PCR under the previous conditions, with a primer mixture containing forward primer 5'-AGCCCAAGATGGATGCGCTCA-3' and reverse primer 5'-CAGTTCTGGTCTCCTGGGAGTCA-3' for VYR and DEC types and reverse primer 5'-TCAAAGCCGCTCTTCCTGAACGT-3' for ERL type. PCR products were gel-purified and TA-cloned into pCR-XL-TOPO® plasmid (Invitrogen).

ED14 inner ear was used to clone variants of BK-DEC, -VYR, and -ERL types using RT-PCR under the previous conditions with a primer mixture. The forward primer, 5'-CGCGGATCCAGCCCAAGATGGATGCGCTCA-3', contained a BamHI (5') site, whereas the reverse primers varied for the different C-term phenotypes; a reverse primer 5'-TGCTCTAGAAGTTCTGGTCTCTGGGAGTCA-3' for BK-DEC and VYR and a reverse primer 5'-TGCTCTAGAGCATCAAAGCGCTCTTCCTGAACGT-3' for BK-ERL, with an XbaI (5') site common to all three. PCR products were purified and cut with BamHI and XbaI at 37°C, overnight. Fragments were gel-purified, and ligated into the BamHI and XbaI sites of pcDNA3.1(+). All cloned BK-VYR, -DEC, and -ERL types were submitted for sequencing (ICBR, University of Florida, Gainesville, FL). All primers were obtained from Integrated DNA Technologies (Coralville, IA) and all enzymes from New England Biolabs (Ipswich, MA).

Real-Time RT-PCR

Total RNA from PD4, 14, and 30, was extracted from cochleae dissected to include the sensory hair cells with supporting cells and excluded the ganglion, modiolus, and lateral wall. The entire cochlea was used for ED14, since we could not cleanly remove the sensory epithelium without retaining other cell types. These tissues were used for quantitative real-time RT-PCR (qRT-PCR). The Chromo4 and DNA Engine Opticon 2 real-time PCR detection system (Bio-Rad, Hercules, CA) was used to reverse-transcribe and amplify each VYR, DEC, and ERL variant of the mouse BK α -subunit, with β -actin serving as an internal control. The reverse primer for each BK variant was designed to anneal uniquely to VYR, DEC, and ERL C-termini. Sequences of primers and TaqMan® minor groove binder (MGB™) probes (Applied Biosystems, Foster City, CA) are shown in Table 1. All probes were labeled with the fluorescent reporter FAM at each 5' end and the quencher TAMRA at the 3' end. qRT-PCR using TaqMan® RNA-to-CT™ 1-Step Kit (Applied Biosystems) was carried out in 10 μ l total reaction volumes, containing 80 ng of total RNA from each stage, 5 μ l of 2 $\times$ TaqMan® RT-PCR Mix, 900 nM each of forward and reverse primers, 50 nM TaqMan® MGB™ probes, and 0.25 μ l of 40 $\times$ TaqMan® RT Enzyme Mix in a 96-well optical tray with caps. The following thermal cycler settings were used: 48°C for 15 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. The experiment was repeated 3X and all samples were run in triplicate. Data were analyzed using the Enumerator Program in the Opticon Monitor Analysis software version 3.1. The threshold cycle (C_t) values of BK-VYR, -DEC, -ERL, and β -actin, were obtained, to determine the quantities of each at different developmental stages and three different regions of PD30 cochleae. Relative quantification was performed using the Delta C_t model. VYR, DEC, ERL, and β -actin were quantified by calculating $2^{\Delta C_t}$ where ΔC_t is the average C_t of VYR, DEC, ERL, normalized to the average β -actin so that $\Delta C_t = C_t$ of each variant – C_t of β -actin. The lowest $2^{\Delta C_t}$ value among VYR, DEC, and ERL types was fixed at one and the others compared as the relative ratio.

Exon RT-PCR

Total RNA was extracted for RT-PCR from tissues as described for qRT-PCR. RT-PCR, which was performed to observe the expression of specific exons, with forward and reverse primers (Table 2) using SuperScript™ One-Step RT-PCR for Long Templates. Mouse β -actin was used as an internal control. Each sample contained 250 ng of total RNA and the volume of each RT-PCR reaction was adjusted to 20 μ l. The thermal cycler was set for: 1 cycle at 50°C for 30 min, 94°C for 2 min, 35 cycles at 94°C for 30 s, 55°C for 30 s, 68°C for 1 min, followed by 1 cycle at 72°C for 8 min. Each PCR product was mixed with 5 μ l of 6x DNA Blue/Orange Loading Dye (Promega, Madison, WI) and 5 μ l volumes were loaded on 1.5% agarose gels (Bio-Rad).

Results

Immuno-electron Microscopy

Immunogold labeling of sensory tissues showed particles in different regions of sensory/neural and supporting cells. Inner hair cells showed lines of particles at the apical neck, bilaterally, as well as small clusters in some mitochondria (Figs. 1A-C). In comparison, outer hair cells showed labeling at the base across from nerve fibers. Immunogold particles were found juxtaposed on both the plasmalemma of the outer hair cell and nerve fiber (Fig. 1D-G). As with inner hair cells, some outer hair cell mitochondria were labeled on the inner mitochondrial membrane (data not shown). In supporting cells, labeling was found at the apex of Deiters' cells in regions opposite the basal portion of the outer hair cells (Fig. 1H, I). In the Figure shown, labeling appeared in electron dense regions of Deiters' cells. Besides nerve fibers, spiral ganglion cell bodies contained mitochondria with immunogold labeling (Fig. 1J). Finally, unexpected labeling with the described antibody, which targets the BK C-terminus, was found in the nucleus of sensory cells. Control samples, prepared without primary antibody did not show immunogold labeling as described above (data not shown).

Cloning of mouse cochlear BK variants

We obtained a total of 27 variants that do not correspond to any known BK variants when compared to previously published cDNAs and proteins found in GenBank (NCBI, Bethesda, MD). A recent study of adult chick cochlea reported 28 variants from a possible 48 (Miranda-Rottmann et al., 2010). Eleven of our variants were obtained from PD14 and PD30 mice and consist of one VYR, six DEC, two ERL, and two truncated variants, which we will refer to as N+C types. Sixteen variants were obtained from ED14 mice and consist of eight VYR, five ERL, and three N+C types. N+C types represent two predicted ORFs, potentially generating N- and C-termini BK from one BK transcript. A total of 22 full-length variants (Acc. Nos. FJ872117; HQ221743 – HQ221763) that include nine VYR, six DEC, and seven ERL, plus an additional five N+C types (HQ221763, HQ529469, HQ529471, HQ529473, HQ529475, HQ529477) were submitted to GenBank (Table 3).

BK exon 1, which is conserved in Mammalia, contains three predicted open reading frames (ORFs): MAN, MSS, and MDA at splice site X1. To clone the full-length mouse BK α -subunit, the forward primer was set for MDA. The reverse cloning primer for full-length BK-VYR and DEC was set just after the stop codon of exon 35a/b, thus covering stop codons for DEC and VYR and amplifying both variants. The reverse cloning primer for full-length BK-ERL was set at the ERL stop codon, which is in an alternatively-spliced exon 33b. Full-length BK variants with each of the three tail types was amplified from total RNA of ED14 inner ears, and PD14, and 30 cochleae by RT-PCR, using these primer pairs. Variants were cloned into vectors and their cDNA was sequenced and translated into amino acids.

Alternatively spliced BK exons

Alternatively spliced exons were revealed in BK variants from mouse ED14 inner ear, and PD14 and 30 cochleae. The alternatively spliced sites, X1 to X7, are shown in relation to amino and carboxy termini, transmembrane and RCK regions, and the Ca^{2+} bowl (Fig. 2). Amino acid sequences that correspond to individually spliced exons at each site $\times$ are illustrated in Figure 3. This exon map (Fig. 3A) depicts all exons incorporated in our twenty-seven BK variants and was determined by the order of the splice donor and acceptor site for each exon, as derived from the chromosome 14 genomic contig (Accession no.: NT_039596).

Site X1, as mentioned previously, contains one exon with three potential start codons within the ORF. Site X2 contains a variant (v2) of the DEC tail type. This variant skips exon 2, which encodes a linker region between the S0 to S1 loop that consists of 54 aa and is included in the region coupling the $\beta 1$ subunit (Liu et al, 2008). The alternative splice site at X3 is between S6 and the RCK1 domain. In this region, the BK transcript is normally composed of exons 8, 9, and 11. We obtained an ERL type variant (v4), cloned from PD30 mouse cochlea that has exons 8, 10, and 11. Exon 10 (also known as 9alt; Soom et al., 2008) is the same length as exon 9, but is spliced between exon 9 and 11. The protein sequence of exon 10 contains 31 aa and is 61% homologous to exon 9 at the aa level. Finally, X4 contains exon 18, a small exon that encodes the four aa, SRKR, whose nucleotides are spliced into three different BK variants (v12, 20, 21) that were all cloned from ED14 mouse inner ear. Variant-12 is an ERL type, whereas variants-20 and -21 are VYR types.

We obtained five types of inserts between exons 20 and 24: ZERO, IYF, STREX, IYS + STREX, and exon 23, all located at splice site X5 between RCK1 and RCK2. ZERO is an insertless variant that skips three exons between exons 20 and 24. Exon 21 encodes IYF (Ile, Tyr, and Phe). STREX is alternatively spliced as exon 22 and contains a cysteine-rich sequence (Xie & McCobb, 1998). IYS (Ile, Tyr, and Ser) + STREX is generated from exons 21 and 22 (STREX). This combination changes the Phe encoded by exon 21 (IYF) to a Ser, producing IYS. Exon 23 includes an SH3 domain-binding motif, PxxPxxP, which interacts with the cortactin SH3 domain (Tian et al., 2006). Of the total reported here, the number of BK variants that contain each insert are: twelve ZERO (v3, 4, 6, 8, 9, 13 - 15, 23 - 25, 27), five IYF (v1, 2, 5, 7, 10 - 11), five STREX (v17 - 20, 22) one IYS + STREX (v21), and three exon 23 (v12, 16, 26) (Table 3). BK-ZERO variants cloned from ED14, PD14, and PD30 contain at least one of the three tail types. BK-IYF variants cloned from PD14 and 30 contain DEC and VYR tails. BK-STREX cloned from ED14 consists of N+C types as well as VYR and ERL tail types. The one IYS + STREX variant is of the VYR tail type. Finally, variants containing exon 23 consist of VYR or ERL tail types.

The alternatively spliced-in exon 29 known as ALCOREX at site X6 in RCK2 between exons 28 and 30 appears just before the Ca^{2+} bowl, which consists of a series of aspartate residues with a high-affinity Ca^{2+} sensor that induces an α to β conformation, regulating Ca^{2+} gating (Kim et al., 2006; Yusifov et al., 2008). ALCOREX consists of 81 bps encoding 27 aa (Fig. 3B) and shows a high sensitivity to alcohol (Pietrzykowski et al., 2008). We obtained clones of four ALCOREX BK variants from PD14 and 30, but not from ED14. The tail motifs of these variants are of the VYR and DEC type, only.

BK has three different types of C-tails, DEC, ERL, and VYR at splice site X7 (Figs. 3A, B). DEC is the longest of the three tails, containing exons 33a, 34, and 35a/b. The addition of exon 34 causes a frameshift, leading to an extremely long exon 35 when compared to the VYR tail type. Moreover, in DEC, exon 35a is longer than 35b by the addition of an alanine, which is a result of a difference in acceptor sites for 35a relative to 35b. The ERL tail is the shortest, as seen in variants 4 and 6. It is encoded by exon 33b, which contains a stop codon

and does not contain any sequence variations. The difference between exons 33a and 33b is that they have different donor sites. Finally, VYR tails have exons 33a and 35a/b, where 35a and 35b encode the sequences NRKEMVYR and KKEMVYR, respectively. These inserts can differ slightly as a result of mutations in exons 33a and 35b that produce SRKEMVYR and KKEVAYR, respectively.

Expression of exons during cochlear development

To clone longer BK variants that include the ATG codon for the methionine of MAN or MSS, we first used 5' RACE using total RNA from PD14 mouse cochlea. The starting reverse primer was set in exon 2 and nested primers were used for 5' RACE to extend into 5' regions. Using this technique, we were able to obtain amplicons containing nucleotides encoding MSS, but not MAN (Fig. 4A); previously, the longest BK extending from MAN was confirmed by 5' RACE using rat cochlea (**Langer** et al., 2003). To detect the nucleotides encoding MAN, we used total RNA from ED14 inner ear, and PD4, 14, 30, and 34 cochleae for RT-PCR. The forward primer was set at the MAN site and the reverse primer, which was common to MSS and MDA, was set in exon 2. Each forward primer of the MSS or MDA site was used as a positive control (Fig. 5A). Amplicons formed when using primer pairs designed for MSS and MDA, but were undetectable for all stages of development when using primer pairs for MAN (Fig. 5A). Qualitatively, BK transcripts encoding MDA were expressed more highly than those encoding MSS at any developmental stage. On PD34, however, transcripts encoding both MSS and MDA decreased compared to their expression at younger ages (Fig. 5A).

The region between exons 8 and 11 at X3 epitomizes some of the diversity found in our BK variants, especially when examining variants-3 and -4 (Fig. 4B). To investigate the variation in this region, RT-PCR was performed using total RNA from different ages, as before, with forward and reverse primers set at exons 8 and 10, respectively (Figs. 4A, B). The ERL type of variant-4 is composed of exons 8 and 10, which encodes the entire BK protein without a stop codon in these exons. Expression of this variant was very faint on ED14 and PD4, but detectable on PD14 and PD34 (Fig. 5C). In contrast, variant-3 belongs to an N+C type configuration, and contains exons 8, 9, and 10 with a stop and start codon in exon 10 and a 37 bps overlap between both codons in exon 10. This configuration predicts two ORFs, suggesting the potential for encoding two different proteins, (Fig. 4B) one at the N-terminus with 357 aa (1071 bps) and the other at the C-terminus with 800 aa (2400 bps). The N-term ends 35 aa after S6, so it does not include the tetramerization domain known as L6, since this region consists of 171 aa and lies between S6 and S7. This variant is expressed on ED14 and downregulated after birth (Fig. 5C). In addition to these two variants, a longer fragment was amplified in the same RT-PCR reaction. This N+C fragment is upregulated on ED14 and PD4, but dramatically decreases on PD14, 30, and 34 (Fig. 5C). To confirm this band, we gel-purified and sequenced both strands, which consisted of exons 8, 9, an intron, and exon 10 (exon 8+9+intron+10). The intron includes a stop codon for the N-terminus region, whereas exon 10 includes a predicted start codon for the C-terminus region. Thus, BK variants that retain an intron between exon 9 and 10 have an N+C type configuration.

Another region of interest is an alternative splice site at X5, where unique exons contribute to variations in BK. RT-PCR was employed to identify STREX (exon 22) and exon 23 transcripts in the mouse ED14 inner ear, and PD4, 14, 30, and 34 sensory epithelia. For STREX, forward and reverse primers were set in exons 20 and 22, respectively (Fig. 4A). STREX was highly expressed on ED14, decreased after birth, especially on PD14 and 30, and increased again on PD34 (Fig. 5B). To determine exon 23 expression, forward and reverse primers were set in exon 23 and 24, respectively (Fig. 4A). Transcripts containing exon 23 were well expressed on ED14, decreased on PD4, increased slightly on PD14 and diminished to low levels in the mature cochlea of PD30 and 34 (Fig. 5B).

An additional insert at X5, exon 24, includes a cluster of ten adenines of BK-VYR, DEC, and ERL tail types with a complete coding sequence (Table 3; Fig 3A). We sequenced this region before sequencing the entire BK clone, because numerous variants showed nine or 11 adenines in our results, thus, producing putative N+C type proteins in two different ORFs. Variants 9, 17, and 18 have nine adenines and variant 19 has 11. Variants with nine adenines have a start codon, ATG, which overlaps a stop codon, TGA, at the underlined nucleotides in exon 24, between RCK1 and RCK2. The stop codon within exon 24 generates N-BK types of 651 aa (1953 bps), 709 aa (2127 bps), and 713 aa (2139 bps) in length (Table 3). The start codon within exon 24 relinquishes C-BK types of 514 aa (1542 bps), 462 aa (1386 bps), and 462 aa (1386 bps) in length. In comparison, variant-19 has 11 adenines in exon 24. This additional adenine causes a predicted stop codon in exon 24 to generate an N-BK of 654 aa (1962 bps) and a predicted start codon in exon 22 to generate a C-BK of 490 aa (1470 bps). There are 71 bps between both types from exon 22 to 24 in this variant. Proteins translated from either 9 or 11 adenines consist of an N-BK that includes the RCK1 domain and is, thus, longer than C-BK, which consists of RCK2 and the remaining C-tail.

Quantification of C-terminal motifs during development and in different regions of the cochlea

BK-VYR, -DEC, and -ERL type C-terminal ends are determined by alternative splicing between exons 33a/b and 35a/b. Each tail motif displays its own biological and physiological function through protein-protein interactions (Ma et al., 2007; Kim et al., 2008; Kim et al., 2009; Chiu et al., 2010). Thus, the difference in C-terminus motifs among BK variants is an important factor in elucidating the diversity of BK channels in the cochlea. Here, we quantify their expression by qRT-PCR for embryonic and postnatal stages and in apical, middle, and basal turns of the cochlea. An ANOVA showed an Age $\times$ Gene effect when comparing gene expression at different ages ($p < 0.00001$). Planned comparisons showed that BK-DEC transcripts were the most highly expressed on PD4, 14, and 30 ($p < 0.0001$), and the least expressed on ED14 ($p < 0.001$), compared to ERL and VYR (Fig. 6). During its greatest period of expression, PD4 and 14, DEC attained levels that were 6 – 7 times greater than VYR or ERL ($p < 0.0001$). However, by PD30, DEC transcript expression was only 2 – 3 times greater than the other tail motifs ($p < 0.001$). Thus, DEC expression increased rapidly after birth and decreased with hearing maturation (PD30). In contrast, on ED 14, VYR expression was ~ 2 and 12 times greater than ERL and DEC types, respectively ($p < 0.0001$). The ERL tail type was the second most highly expressed variant on ED14, with expression ~ 7 times greater than DEC ($p < 0.0001$). Postnatally, ERL and VYR were equal in expression on PD4 and 30 ($p > 0.05$) and only showed a difference on PD14 ($p < 0.05$).

To determine any differences in expression along the tonotopic axis, tail motifs were quantified for sensory epithelia cut equally into regions containing the base, middle, and apex (Fig. 7A). An ANOVA showed a Region $\times$ Gene effect when measuring the expression of the three different tail types across the different turns ($p < 0.0001$). Planned comparisons showed that BK-DEC had the greatest expression in all regions, relative to ERL and VYR ($p < 0.0001$), especially in the middle turn where DEC expression was 1 - 2 times greater than in apical and basal turns ($p < 0.0001$). In contrast, the expression level of VYR showed no significant change among the three regions; although, VYR expression was greater than ERL in apical, and especially in middle regions ($p < 0.001$), where ERL was barely expressed. BK-ERL showed the greatest amount of expression in basal regions, where it was the second most highly expressed transcript ($p < 0.001$) (Fig. 7B).

Discussion

The BK channel in mammalian cochlear hair cells shows a localization pattern that is dependent on hair cell type. Inner hair cells have immunogold-labeled BK channels at the apical neck of the cell in supranuclear regions of the plasmalemma. This placement is similar to a previous description (Pyott et al., 2004), using a different antibody for confocal microscopy. Presently, we report the absence of BK channels near synaptic regions of inner hair cells; although we have tried several antibodies, we have not completed a survey along the entire tonotopic length. In contrast, immunogold labeling was found at the base of the outer hair cells, labeling that appears juxtaposed in the nerve and sensory cell. This labeling appeared in the absence of any clear synaptic figures. In addition, we report immunogold labeling in mitochondria of both hair and ganglion cells, further suggesting the diversity of the BK channel, at least with regard to the C-terminus, which is represented here as a function of the antibody targeting this region. We did not find labeling in all mitochondria, suggesting that expression may be limited to a subpopulation. An explanation of the labeling found in the nucleus is uncertain at this time, although functional ion channels are expected in the nuclear envelope, which contains various channels including BK (Yamashita et al., 2006; Fedorenko et al., 2010). An alternate scenario may be that the antibody is labeling some truncated form of BK C-terminus. C-BK function may be similar to the C-terminus of Cav1.2, an L-type voltage-gated Ca^{2+} channel that reportedly acts as a transcription factor in the nucleus (Gomez-Ospina et al., 2006). The presence of at least two K^{+} channels in Deiters' cells was suggested previously, although the types of channels are still in dispute, as presently, they are reported as charybdotoxin sensitive Kv1.3 or 1.5 channels (Nenov et al., 1998; Szucs et al., 2006). However, channels may differ with region and even Deiters' cell types (Szucs et al., 2006).

The full-length variants reported here differ when compared to both vertebrate and non-vertebrate *Kcnma1* genes curated in GenBank. Twenty-two variants encoding the complete BK protein were cloned and include inserts at splice sites X1-7 with C-terminus tails ending in VYR, DEC, and ERL. An additional five variants are shown that are truncated as N-BK and C-BK types, as a result of stop and start codons in splice sites X.

Splice site X1 has a single ORF that has several start codons defining exon 1. The ORF beginning with MDA showed the greatest amount of expression for all developmental stages, relative to those encoding MAN or MSS. In comparison, MSS was expressed highly at first but decreased on PD34, whereas MAN was faint or undetectable during any stage in development, and thus may not be an actual initiation site for BK translation in the mouse cochlea. The channel sensitivity of BK constructs beginning with MDA in HEK293 cells is functionally similar to native BK in GH4C1 cells, a rat pituitary cell line (Erxleben et al., 2002). Thus, the methionine in MDA is considered to be a major starter of the BK protein in cochlea. In addition to X1, the N-terminus incorporates splice site X2 where one finds exon 2. All of our variants have exon 2, with the exception of variant-2, a DEC tail type with an alternatively spliced-out exon 2. When present, this exon forms part of the S0-S1 linker and contributes to coupling with $\beta 1$ subunit transmembrane (TM) helices, TM1 and TM2 (Liu et al., 2008). A lack of exon 2 would affect α -subunit gating via β -subunit modulation.

Variant-4, an ERL tail type has exons 8, 10, and 11 between S6 and S7 at X3. BK variants with exon 10 were cloned previously from a human prostate cancer cell line (LNCaP) and found in mouse brain, where they fine-tune neuronal excitability (Soom et al., 2008). Unlike exon 9, exon 10 changes BK gating properties by increasing Ca^{2+} sensitivity (Soom et al., 2008). Exon 10, cloned from PD30 cochlea, was visible after birth (PD4), increased to just after the onset of hearing (PD14), then decreased on PD30 and 34. Splicing-in of either exon

9 or 10 may be required to initially help regulate the onset of hair cell/neural excitation, but plays a lesser role with further development.

At site X3, we identified sequences that define two N+C type variants and one RT-PCR product. N+C variants may encode two predicted proteins that stand alone, an N-terminus (N) BK that includes the extracellular N-terminal end with transmembrane regions and a C-terminus (C) BK that includes the intracellular portion of BK. Thus, variant-3, may be a putative bicistronic structure of the N+C type. This configuration is the result of exon 10, which contains a stop codon for N-BK and a start codon for C-BK in two different ORFs. While polycistronic transcripts are more plentiful in prokaryotes, they are found in eukaryotes. For example, the Small Nuclear Ribonucleoprotein N (*SNRPN*) gene in humans, which underlies the developmental neurobehavioral disorder, Prader-Willi syndrome, contains an upstream ORF that produces a second transcript known as *SNRPN* Upstream Reading frame (*SNURP*) (Gray et al., 1999). Moreover, functional evidence suggests that a truncated form of BK, which lacks the entire C-terminus, still shows wild type Ca^{2+} sensitivity (Piskorowski et al., 2002). A recent study, however, suggests a second alternative in that truncated N-BK modulates BK variants, such as ZERO, by slowing activation and increasing membrane expression, while showing no activity on its own (Poulsen et al., 2011). These reported variants extend 92 and 188 aa downstream of S6. While the function of these truncations in the cochlea are unknown, they warrant further investigation, given that a similar truncation, resulting from a stop codon in exon 10, was reported recently in chick cochlea (Miranda-Rottmann et al., 2010).

The ATG codon in exon 10 of variant-3 may be recognized as an initiation codon for the translation of a C-BK variant that forms independently from the BK gene. This N+C variant was highly expressed on ED14, decreased after birth, and was barely detectable by PD30. Its importance is further underscored by evidence suggesting that the large C-terminus of BK may interact with over a hundred proteins through scaffolding, trafficking, or phosphorylation (Kathiresan et al., 2009). In addition, as with Cav1.2, C-BK function may act as a transcription factor in the nucleus (Gomez-Ospina et al., 2006). Finally, we amplified within X3 a third RT-PCR fragment with over 300 bps that included an intron (8+9+intron+10). A recent report cited the retention of introns after exons 19 and 23 in cochlear BK transcripts (Miranda-Rottmann, 2010). Unfortunately, we found only a PCR product and did not obtain BK variants that retain the intron between exons 9 and 10. Translation of this PCR product showed both a stop codon in the intron and a start codon in exon 10 of the same frame, suggesting putative N- and C-BK proteins, respectively. Moreover, there is a putative transcription factor-binding site in the intron. This exon with intron retention is similar to myo-inositol-3-phosphate synthase γ and δ mRNAs (Seelan et al., 2009). The expression level of this product was greatest on ED14 and PD4, but decreased by PD30.

Variants 12, 18, 20, and 21 have a short insert at X4 that is exon 18, which encodes the aa sequence SRKR. This sequence has been extensively described in both turtle and chick, since it alters channel kinetics and Ca^{2+} sensitivity (Jones et al., 1999a, b; Rosenblatt et al., 1997; Ramanathan et al., 1999).

Single adenine errors, in a polyadenine string of 10 adenines, sometimes appear as an addition or deletion in exon 24 at splice site X5 of BK transcripts. Thus, the potential N+C types generated are not the result of alternative splicing. Presently, it is unclear how mutations in adenine number occur in exon 24, but there is a precedent as they appear at a rate of approximately 5% and cause diseases such as canine cyclic neutropenia (Linton et al., 1997; Benson et al., 2004). These errors may be the result of nucleotides misincorporated and missed during proofreading by RNA polymerase II and transcription

elongation factor S-II (Thomas et al., 1998; Koyama et al., 2003). In addition, they may be due to slippage during the transcriptional reading of long stretches of adenines, sequences that may be selected against in evolution (Linton et al., 1997), but which, nevertheless, can appear as phenotypic mutations.

Included within splice site X5 is the extensively reported STREX variant, which contributes to hair cell tuning and tonotopic organization in turtles (Jones et al., 1999a). BK-STREX was first identified in rat chromaffin cells as a cysteine rich 59 aa insert (Saito et al., 1997) spliced-in as exon 22. STREX has a high affinity for the cell membrane via palmitoylation of evolutionarily conserved cysteine residues (Tian et al., 2008). The inclusion of this exon in BK can alter both expression and sensitivity to phosphorylation. Expression is affected by stress and sex hormones, such that exposure of cells to certain hormones induces the expression of this splice variant as opposed to the variant ZERO. For example, the adrenal androgens, dehydroepiandrosterone, androstenedione, and testosterone, increase STREX, whereas glucocorticoids decrease STREX (Lai et al., 2002; Xie & McCobb, 1998; Mahmoud & McCobb, 2004). STREX transcripts also show changes in expression with age, as in the CNS where STREX is upregulated in the embryo and downregulated after birth, with the least amount of expression in PD35 brain (MacDonald et al., 2006). However it is not known if hormones have an effect on STREX expression in the brain. In chick hair cells, STREX transcripts increased at least two fold from *in ovo* to post-hatching (Kim et al., 2010), whereas immunolocalized protein in the cytoplasm is observed as early as ED11 (Li et al., 2009). All three of our STREX variants, one ERL and two VYR types, were cloned from ED14 cells, and not from PD14 or 30. As in other embryonic systems, but unlike chick, STREX transcripts were highly expressed on ED14 and PD4, but decreased significantly at PD14. The caveat, however, is that STREX expression returns on PD34 as mice reach puberty around 28 to 49 days-of-age, suggesting the mouse cochlea may be more sensitive than brain to hormonal changes on PD34. Is there a relation to functional BK channels in hair cells? BK channels in the plasmalemma of hair cells are functional by PD12-14 in mice (Kros et al., 1998). The inexplicable decrease of STREX at PD14 coincides with functional expression of both intra and extra-cellular Ca^{2+} -activated BK channels, but parallels the continued expression of exon 10 and 23. Whether this decrease is reflected at the protein level would depend on the half-life of the channel. Thus, these relationships need further study to clarify their contribution to functional inner and outer hair cell channels. Nonetheless, similar to exons 10 and 23, STREX may contribute to organizing the embryonic inner ear, since both increase BK Ca^{2+} sensitivity (Saito et al., 1997; Hanaoka et al., 1999; Chen et al., 2005).

Exon 23 at X5, is inserted between exon 20 and 24 instead of STREX. Both transcripts are expressed similarly on ED14. Unlike STREX after birth, however, transcripts containing exon 23 continue expression during critical periods of hearing maturation, then decline only on PD30 and 34, the latter of which marks the time when STREX turns on again. A portion of exon 23 encodes one of three putative PxxPxxP SH3 binding motifs that interact with the cortactin SH3 domain (Tian et al., 2006). Cortactin binds to actin and promotes cytoskeletal remodeling (Ammer & Weed, 2008). This dynamic occurs in hair cells during a period when BK expression includes exon 23 (Weaver et al., 1994; Vago et al., 1996).

The three BK tail phenotypes VYR, DEC, and ERL form through alternative splicing and may regulate BK function and localization. These types were reported in cochlea of rat and chick (Langer et al., 2005; Kim et al., 2010; Miranda-Rottmann et al., 2010). Our results show that of the three, DEC is more highly expressed in mouse after birth, as it is in chick (Kim et al., 2010), whereas ERL and VYR decrease after birth. Ironically, when expressed in heterologous systems, BK-VYR and ERL show the greatest amount of plasmalemmal expression compared to BK-DEC (Ma et al., 2007; Chiu et al., 2010; Ridgway et al., 2009),

which is retained intracellularly (e.g., endoplasmic reticulum) by interacting with partners such as protein phosphatase 2A (PP2A-A) (Ma et al., 2007). Given that the entire cochlea was used at ED14, the initial high expression of ERL and VYR may be a reflection of developing neurons or other cell types. But the inclusion of other tissue types does not explain increased DEC-expression after birth. This increase could be the result of expression of other proteins, since growth factors and BK β -subunits acting as chaperones, can increase BK-DEC plasmalemma expression (Kim et al., 2007a, b). However, increased DEC expression could reflect intracellular expression in the mitochondria or possibly the nuclear envelope (Yamashita et al., 2006; O'Rourke, 2007; Kathiresan et al., 2009). This type of localization is regulated by exon 34, which encodes nine amino acids, ATRMTRMGQ, and with 35a/b contains a trafficking signal for the inner mitochondrial membrane (Siemen et al., 1999), potentially including those of murine hair cells (Kathiresan et al., 2009). If so, BK-DEC may have a role in the type of Ca^{2+} signaling that stabilizes the mitochondrial membrane (Kang et al., 2007). If we presume this scenario, the increase in DEC may correlate with a reported increase in energy metabolism with the onset of hearing (Armour et al., 2001). Thus, BK may not only be a mediator of sound stimuli, but also a positive regulator of cell growth and metabolism (Weaver et al., 2004).

Our data demonstrate the extent of BK variant diversity during development and in different regions of the cochlea. The overall expression level of BK is not much different between embryonic and postnatal stages. However, exons such as 10, STREX, and 23 show interesting changes in expression during periods of embryogenesis, hearing onset, and puberty. Moreover, tail variants, such as BK-VYR and -ERL, are expressed decidedly on ED14, whereas BK-DEC becomes predominant after birth. The early embryonic expression of variants may reflect a role in cochlear organization, rather than hearing, since hearing in mouse begins towards PD10. This hypothesis is buttressed by data that show putative BK partners that have a metabolic role (Kathiresan et al., 2009). Thus, future studies might consider these variants within a framework in understanding the molecular mechanisms of cochlear development.

Acknowledgments

This study was supported by NIH/NIDCD grant DC004295 to BS.

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Figure 1. Localization of the BK channel in sensory, neural, and supporting cells

(A) Immunogold labeling of BK, near the apical neck (inset) of an inner hair cell, is seen in both the sensory and supporting cell plasmalemma at a (B) higher magnification. (C) Gold particles in the inner mitochondrial membrane of an inner hair cell. Low magnification of outer hair cells (D, F) shows nerve fibers at the base (inset). (E, G) A higher magnification of this region reveals juxtaposed gold particles lining the membranes of both the nerve and outer hair cells. Gold particles also are seen in the nucleus of the outer hair cell. (H) At low magnification, the electron dense region (arrows) in a Deiters' cell, encapsulating the base of an outer hair cell, shows labeling at the membrane in a higher magnification. Particles also are seen at the basal membrane of the outer hair cell. (J) Labeling in the mitochondrion of a myelinated ganglion cell. The dark flocculent material in the nucleus of F and H is precipitated KMn_4 stain. *Abbreviations:* DC, Deiters cell; IHC, inner hair cell; Mito, mitochondrion; Myl, myelin; N, nerve; Nuc, nucleus; OHC, outer hair cell.

Figure 2. The BK channel

A. Diagram of the BK α -subunit, showing the N-terminus (NH_2) that projects into the extracellular space and a C-terminus that constitutes approximately 70% of the protein. The transmembrane region of BK contains seven hydrophobic domains, S0-S6, with a pore region (P) between S5 and S6. The long cytoplasmic tail contains four hydrophobic domains, S7-S10. Regions S7-S8 and S9-S10 are within the Regulators of Conductance for K^+ (RCK) 1 (red) and RCK2 (orange) domains, respectively. The Ca^{2+} bowl (blue arrow) is a high affinity Ca^{2+} binding site that lies within RCK2. Unique splice sites, X1 – X7, (red arrows) contain alternatively spliced-in or -out exons.

Figure 3. Exons of the mouse BK channel gene, *kcnma1*

(A) A total of 37 exons are derived, based on our 27 full-length variants and using the chromosome 14 genomic contig (Accession no.: NT_039596) as a template for analysis. Odd (yellow) and even (blue) numbered exons are represented across splice sites X1-X7, where the length of each colored box implies the proportional length of each exon. S0-S10 with the pore region is shown with exons that correspond to these regions. Exon 1 has three predicted start codons (M), where the gradations from gray to black represent least to greatest expression for each codon. Putative start codons (M), which generate C-BK type truncated variants, are found in the C-terminus at exons 10, 22, and 24. Putative stop codons (*), which generate N-BK type truncated variants, are found at exons 10 and 24. Exon 24 contains a cluster of 9, 10, or 11 adenines (xA) that can generate N+C type truncated variants. STREX, STREXalt, and ALCOREX variants are found at exons 22, 23, and 29, respectively. Exons 33a and b are spliced with a common acceptor but different donor sites, so that exon 33b is not only longer than 33a, but also contains a stop codon (*) for the ERL type C-terminus. Exons 35a and b share a common exon, however exon 35a contains the nucleic acid sequence, CAG, before the acceptor site for 35b so that exon 35a is 3 bps longer than 35b. Exons 35a and b include stop codons (*) for both DEC and VYR tail types. The DEC type is determined by the splicing-in of exon 34. Mutations are also found in exons 33a (m33a) and 35b (m35b) that generate alternative VYR tail variants. (B) Amino acid sequences of the spliced-in exons at sites X1-7. Each splice site contains numbered exons that are splice variants (bold black) with their flanking exons (gray). The number of asterisks, in site X3, corresponds to the number of stop codons contained in the exon or intron.

Figure 4. Priming sites and exon diversity

(A) Priming site locations (arrows) and forward and reverse primers used to obtain the RT-PCR products of BK exons. Primer pairs were set to detect variations in exons 1, 10, 22, and 23. Exons 1 (MAN/MSS/MDA), 8, 20, and 23 forward primers are coupled with exons 2, 10, 22, and 24 reverse primers, respectively. RT-PCR for each exon was performed using total RNA from ED14 cochlea and PD4, 14, 30, and 34 sensory epithelia. (B) Illustrations of exon diversity by alternative splicing between exons 8 and 11. Exon 8 forward and exon 10 reverse primers were set to detect variations in this region by RT-PCR. BK variant-4, an ERL tail type, contains exon 10 instead of 9. Variant-3 encodes a truncated N- and C-BK, because of the splicing-in of both exon 9 and 10. The putative start and stop (*) codons for C- and N-BK, respectively, are in exon 10. The RT-PCR product, 9+intron+10, has a stop codon (*) in the intron and a start codon in exon 10, thereby encoding putative N- and C-BK, respectively. Vertical peaks indicate exclusion of exon and/or intron.

Figure 5. Qualitative analysis of exons 1, 8, 9, 10, 22, and 23 during development
(A) RT-PCR products (bps) from exon 1 encoding MSS (470) and MDA (348) are highly expressed during development, whereas the product encoding MAN (547) is barely detectable. **(B)** RT-PCR products for exon 22 (226/235*) and exon 23 (137) reveal that exon 23 expression decreases with age. In comparison, exon 22 transcripts decrease from ED14 to PD30, but upregulate again on PD34. The exon 22 (235) product contains 9 bps of exon 21, which encodes IYF. β-actin was used as an internal control. **(C)** RT-PCR products (bps) for 8+9+intron+10 (354), 8+9+10 (257), and 8+10 (165) show that the first two products upregulate on ED14, but decrease with age. In contrast, 8+10 shows the least amount of expression on ED14. These exons introduce truncated N- and C-BK variants.

Figure 6. Quantification of tail variants during development

Total RNA extracted from ED14, PD4, 14, and 30 mice was subjected to RT followed by qPCR, using primers designed to anneal to BK tail variants ERL in exon 33, and VYR and DEC in exon 35. DEC showed the least amount of expression on ED14, but was the most highly expressed type on PD4, 14, and 30. Gene expression within each age group was compared to the minimum (fixed as one) to ascertain statistical significance, which was determined by using an ANOVA ($p < 0.0001$) with planned comparisons (* $p \leq 0.05$, ** $p \leq 0.001$). Error bars define the standard deviation.

Figure 7. Quantification of tail variants from different turns of the cochlea

(A) A schematic representation of the CBA/J mouse cochlea shows an approximation of the three parts used for the qRT-PCR experiments examining tail types on PD30. The tonotopic organization used in the diagram was derived from the data of Müller et al. 2005. (B) Total RNA was extracted from the apex, middle, and basal parts of PD30 mouse sensory epithelium and employed for qRT-PCR, using amplification primers specific for VYR, DEC, and ERL. Amplicons of each tail motif from qRT-PCR experiments were normalized to mouse β -actin. The DEC tail type was the most highly expressed in all three turns, whereas the second most highly expressed was VYR in the apex and middle, and ERL in the base. Gene expression within each age group was compared to the minimum (fixed as one) to ascertain statistical significance, which was determined by using an ANOVA ($p < 0.0001$) with planned comparisons (* $p \leq 0.05$, ** $p \leq 0.001$). Error bars define the standard deviation.

Table I
Primers and probes used to obtain BK variants with qRT-PCR

BK reverse primers were designed from tail motifs, VYR, DEC, and ERL of variants 1, 5, and 4, respectively. The same forward primer was paired with all reverse primers for each specific tail type. Tail types were amplified and analyzed using the Chromo 4 real time PCR system. Twenty-one bps of PCR product were labeled with a minor groove-binding (MGB) probe tagged with the fluorescent reporter FAM at the 5' end. Nucleotide positions of primers and probe for the internal control, mouse β -actin, were determined from GenBank Acc. No. NM_007393. All PCR products were confirmed by direct sequencing with double strands before running qRT-PCR.

Primers and MGB probe	Sequence (5' to 3')	Nucleotide position
Forward primer	CACTCCTCACAGTCGTCCAGT	3307-3327 in Variant-1 (VYR) 3307-3327 in Variant-5 (DEC) 3226-3246 in Variant-4 (ERL)
Reverse primer	CTGTAAACCATTCTTTTCTGTTTGTCGCGG CCCATTCTAGTCATCCTTGTGTC TCAAAGCCGCTCTTCCTGAACGT	3399-3431 in Variant-1 (VYR) 3415-3357 in Variant-5 (DEC) 3335-3357 in Variant-4 (ERL)
MGB probe	TCCACAGCAAATCGGCCGAAC	3358-3378 in Variant-1 (VYR) 3358-3378 in Variant-5 (DEC) 3277-3297 in Variant-4 (ERL)
β -actin forward primer	GCTCTGGCTCCTAGCACCAT	1034-1053*
β -actin reverse primer	GCCGGACTCATCGTACTCCT	1158-1177*
β -actin MGB probe	TGTGGATTGGTGGCTCCAT	1095-1113*

Table II
Primer sequences and sites used to obtain exon products with RT-PCR

Forward and reverse primers were designed for specific exons in our variants. Except for exons 10 and 22, forward and reverse primers were set in exons next to each other to detect the appropriate-sized band. In exon 10, forward and reverse primers were set in exons 8 and 10, respectively, resulting in the amplification of three different length fragments. For exon 22, the forward primer was set in exon 20 because exon 21 is short (9bps). Each fragment was gel-purified and sequenced with double strands. Mouse β -actin was used as an internal control.

Product name	Primer sequence (5' to 3')	Primer site	Size (bps)
MAN	Forward: TGCTACGTATGGCAAACGGT Reverse: AGGACTCTGCCAGTCAGTGTCT	Exon 1 Exon 2	547
MSS	Forward: CTTAGAATGAGCAGCAATATCCACGC Reverse: AGGACTCTGCCAGTCAGTGTCT	Exon 1 Exon 2	470
MDA	Forward: CAAGATGGATGCGCTCATCA Reverse: AGGACTCTGCCAGTCAGTGTCT	Exon 1 Exon 2	348
Exon 10	Forward: CAGGCACTTACGTACTGGGA Reverse: AGCAGCAATTTCGGGCACGT	Exon 8 Exon 10	354/257/165
Exon 22	Forward: CATGATGACGTCACAGATCCCA Reverse: AGGCACGGAACTGGTGGAGCA	Exon 20 Exon 22	226/235
Exon 23	Forward: GACTCCCAATTCTCCACACGT Reverse: AGGGGTCATGCCTCATCAGCT	Exon 23 Exon 24	137

Table III
Features of BK variants cloned from ED14, and PD14 and 30

Variants cloned from mouse cochlea at different ages. Variants are identified by tail or bicistronic type, and exon usage, in addition to cloning vector and site, age at cloning, and total number of base pairs (bps) and amino acids (aa). Variant 3 is an N+C type, with predicted stop and start codons on exon 10 between S6 and S7, resulting in the translation of two proteins, an N- or C-BK. Variants 9, 17, and 18 have a string of 9 or 11 adenines on exon 24 between S8 and S9. This string contains a predicted start and stop codon that generates an N- and C-BK, containing RCK1 or RCK2 with the remaining C-terminus, respectively. With 9 adenines, both the predicted start and stop codons appear on exon 24. Variant 19 has 11 adenines with a predicted start codon on exon 22 and a stop codon on exon 24 to generate a C- and N-BK, respectively. ZERO variants are unspliced between exons 20 and 24.

Variant	Type	Exon usage	Age	bps	aa	Cloning vector and sites (5'-3')
1	VYR	Exon21	PD30	3590	1142	pcDNA3.1, BamHI/BgIII ^{d)} -XbaI
2	DEC	Exon21	PD30	3379	1115	pcDNA3.1, BamHI/BgIII-XbaI
3	N+C	ZERO [#] in C	PD30	3595	357(N)+800(C)	pcDNA3.1, BamHI/BgIII-XbaI
4	ERL	ZERO	PD30	3357	1113	pcDNA3.1/NT-GFP-TOPO, T-A ^{b)}
5	DEC	Exon21	PD30	3619	1195	pcDNA3.1, BamHI/BgIII-XbaI
6	ERL	ZERO	PD14	3348	1113	pCR-XL-TOPO, T-A ^{b)}
7	DEC	Exon21	PD14	3538	1169	pCR-XL-TOPO, T-A
8	DEC	ZERO	PD14	3607	1192	pCR-XL-TOPO, T-A, Reversed ^{c)}
9	N+C	ZERO in N	PD14	3525	651(N)+514(C)	pCR-XL-TOPO, T-A
10	DEC	Exon21	PD14	3619	1196	pCR-XL-TOPO, T-A, Reversed
11	DEC	Exon21	PD14	3535	1168	pCR-XL-TOPO, T-A, Reversed
12	ERL	Exon23	ED14	3449	1145	pcDNA3.1, BamHI-XbaI
13	VYR	ZERO	ED14	3499	1113	pcDNA3.1, BamHI-XbaI
14	ERL	ZERO	ED14	3353	1113	pcDNA3.1, BamHI-XbaI
15	ERL	ZERO	ED14	3353	1113	pcDNA3.1, BamHI-XbaI
16	ERL	Exon23	ED14	3440	1142	pcDNA3.1, BamHI-XbaI
17	N+C	Exon22 in N	ED14	3526	709(N)+462(C)	pcDNA3.1, BamHI-XbaI
18	N+C	Exon22 in N	ED14	3684	713(N)+462(C)	pcDNA3.1, BamHI-XbaI
19	N+C	Exon22 in N	ED14	3518	654(N)+490(C)	pcDNA3.1, BamHI-XbaI
20	VYR	Exon22	ED14	3685	1175	pcDNA3.1, BamHI-XbaI
21	VYR	Exon21 + 22	ED14	3694	1178	pcDNA3.1, BamHI-XbaI
22	ERL	Exon22	ED14	3527	1171	pcDNA3.1, BamHI-XbaI

Variant	Type	Exon usage	Age	bps	aa	Cloning vector and sites (5'-3')
23	VYR	ZERO	ED14	3498	1112	pcDNA3.1, BamHI-XbaI
24	VYR	ZERO	ED14	3496	1112	pcDNA3.1, BamHI-XbaI
25	VYR	ZERO	ED14	3496	1112	pcDNA3.1, BamHI-XbaI
26	VYR	Exon23	ED14	3585	1142	pcDNA3.1, BamHI-XbaI
27	VYR	ZERO	ED14	3499	1113	pcDNA3.1, BamHI-XbaI

a) A BamHI site in pcDNA3.1 vector was complementary ligated to a BglII site attached to the 5' end of BK inserts.

b) The 5'-A and T-3' of the BK PCR products were cloned into Thymine-Adenine (TA) vectors through 5'-T/A and T/A-3' ligation.

c) BK variants were generated in reverse against the Lac promoter in pCR-XL-TOPO vector.