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***Slc26a4*-Insufficiency Causes Fluctuating Hearing Loss and Stria Vascularis Dysfunction**

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Abstract

SLC26A4 mutations can cause a distinctive hearing loss phenotype with sudden drops and fluctuation in patients. Existing *Slc26a4* mutant mouse lines have a profound loss of hearing and vestibular function, with severe inner ear malformations that do not model this human phenotype. In this study, we generated *Slc26a4*-insufficient mice by manipulation of doxycycline administration to a transgenic mouse line in which all *Slc26a4* expression was under the control of doxycycline. Doxycycline was administered from conception to embryonic day 17.5, and then discontinued. Auditory brainstem response thresholds showed significant fluctuation of hearing loss from 1 through 3 months of age. The endocochlear potential, which is required for inner ear sensory cell function, correlated with auditory brainstem response thresholds. We observed degeneration of stria vascularis intermediate cells, the cells that generate the endocochlear potential, but no other abnormalities within the cochlea. We conclude that fluctuations of hearing result from fluctuations of the endocochlear potential and stria vascularis dysfunction in *Slc26a4*-insufficient mouse ears. This model can now be used to test potential interventions to reduce or prevent sudden hearing loss or fluctuation in human patients. Our strategy to generate a hypomorphic mouse model utilizing the tet-on system will be applicable to other diseases in which a hypomorphic allele is needed to model the human phenotype.

Keywords

Conditional; doxycycline; endocochlear potential; fluctuation; hearing loss; hypomorph; inducible; *SLC26A4*; stria vascularis; transgenic; vestibular aqueduct

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Introduction

SLC26A4 encodes an 86-kDa transmembrane anion exchanger called pendrin. Mouse *Slc26a4* is expressed in the inner ear, thyroid, kidney, lung, and several other organs (Alesutan et al., 2011; Everett et al., 1997; Rehman et al., 2014). Pendrin mediates $\text{Cl}^-/\text{HCO}_3^-$ exchange in the developing inner ear and is required for proper endolymphatic pH and volume (Choi et al., 2011; Wangemann et al., 2007). *SLC26A4* mutations cause Pendred syndrome (PS), an autosomal recessive disorder comprised of goiter, hearing loss (Pendred, 1896) and enlargement of vestibular aqueduct (EVA) (Reardon et al., 2000). EVA is a common inner ear malformation detected in up to 20% of children with sensorineural hearing loss (Morton and Nance, 2006). However, many cases of EVA are not associated with thyroid goiter (PS) or *SLC26A4* mutations.

Patients with EVA can have hearing loss whose onset is postlingual with severity that ranges from mild to profound with variable audiometric configurations (King et al., 2010). Progressive or fluctuating hearing loss is commonly observed and may be precipitated by minor head injury or barotrauma in some patients (Griffith and Wangemann, 2011). Although some studies identify associations of inner ear morphology with hearing levels or prognosis (Campbell et al., 2011; Dahlen et al., 1997), the associations may be epiphenomenal reflections of underlying correlations with age, genotype or other factors. When underlying genotypic and phenotypic correlations are accounted for, we could not detect an association of the presence of a cochlear anomaly with severity of hearing loss in ears with EVA (King et al., 2010). Moreover, most studies have found no correlation of the size of the vestibular aqueduct with degree of hearing loss in ears meeting the diagnostic criteria for EVA originally proposed by Valvassori and Clemis (Griffith et al., 1996; King et al., 2010). Therefore gross morphogenetic anomalies seem unlikely to be the direct cause of hearing loss in EVA.

Many authors believe that endolymphatic hydrops, a pathologic enlargement of the scala media fluid compartment that bathes the apical mechanosensory surface of neurosensory hair cells, underlies fluctuating hearing loss in auditory-vestibular disorders such as Meniere's disease (Schuknecht et al., 2010). However, a controlled study of normal and diseased human temporal bones indicates that endolymphatic hydrops is an associated epiphenomenon that is not a direct cause of hearing loss in Meniere's disease (Merchant et al., 2005). There are no similar published studies of patients with isolated EVA to test the hydrops hypothesis and, therefore, the pathogenesis of fluctuating hearing loss in EVA and other disorders remains enigmatic.

The endocochlear potential (EP) is required for cochlear hair cells to transduce the mechanical stimulus of sound vibrations to an electrical signal comprised of hair cell depolarization, followed by glutamate release at the basal hair cell presynaptic membrane, and excitation of the afferent auditory nerve (Von Békésy, 1952; Wangemann, 2006). The EP is approximately 80 to 100 mV generated by the stria vascularis (Wangemann, 2006). It is widely accepted that a decreased EP impairs cochlear function and sensorineural hearing (Wangemann, 2006).

The stria vascularis is comprised of three layers: marginal, intermediate and basal (Fig. 1C) (Jahnke, 1975). The basal layer is comprised of a tight junction barrier connecting epithelial cells with an inner membrane facing the intrastrial space, and an outer membrane contacting the spiral ligament (Fig. 1C). The inner membrane of basal cells is connected to stria intermediate cells via gap junctions, such that intermediate cells are electrochemically coupled to the inner membrane of the basal cell barrier (Kikuchi et al., 1995; Lautermann et al., 1998; Xia et al., 2001; Xia et al., 1999). Gap junctions on the outer membrane of basal cells electrochemically couple them to fibrocytes of the spiral ligament. This stria architecture is required for generation of a normal endocochlear potential (Wangemann, 2006).

Potassium ions circulate between endolymph and perilymph (Wangemann, 2006). Potassium flows from endolymph through hair cells and into perilymph from which it enters spiral ligament fibrocytes and travels via gap junction networks to stria vascularis basal cells and intermediate cells (Marcus et al., 2002; Takeuchi et al., 2000). The endocochlear potential is a K^+ equilibrium potential that is generated by the KCNJ10 (Kir 4.1) channel located in the highly convoluted plasma membrane of intermediate cells facing the intrastrial space (Ando and Takeuchi, 1999; Marcus et al., 2002; Nin et al., 2008; Takeuchi et al., 2000). KCNJ10 releases K^+ across this large membrane surface area into the intrastrial fluid, from which K^+ is taken up via the $Na^+/2Cl^-/K^+$ co-transporter SLC12A2 (NKCC1) and Na/K ATPase in the equally convoluted basolateral membrane of marginal cells (Crouch et al., 1997; Dixon et al., 1999; Mizuta et al., 1997; Wangemann et al., 1995). Marginal cells secrete K^+ into the scala media by an apical membrane potassium channel comprised of KCNQ1 (Kv1qt1) and KCNE1 (Isk or minK) (Marcus et al., 1985; Takeuchi et al., 2000; Wangemann et al., 1995).

Mice that are homozygous for a targeted deletion allele (*Slc26a4*^{-/-}) of *Slc26a4* have profound loss of hearing and vestibular function, and massively enlarged endolymphatic spaces throughout the entire inner ear (Everett et al., 2001). The hearing loss in this *Slc26a4*-null model is associated with an abolished endocochlear potential (Royaux et al., 2003; Wangemann et al., 2004). There are numerous associated pathologic changes, beginning with scala media expansion and endolymphatic acidosis at embryonic days 14.5 and 15.5 (E14.5 and E15.5) (Everett et al., 2001; Kim and Wangemann, 2010; Kim and Wangemann, 2011), followed by oxidative stress (Singh and Wangemann, 2008), macrophage invasion (Jabba et al., 2006), increased pigment formation (Jabba et al., 2006; Wangemann et al., 2004), and loss of KCNJ10 expression in the stria vascularis (Wangemann et al., 2004; Wangemann et al., 2007). The rapid development and severity of these abnormalities, as well as the severe morphogenetic malformations do not model the less severe phenotypes frequently observed in many EVA patients (Choi et al., 2009; Griffith et al., 1996; King et al., 2010; Pryor et al., 2005).

We recently reported a mouse model that more closely approximates the human phenotype (Choi et al., 2011). This model utilizes genetically unlinked effector (Tg[E]) and responder (Tg[R]) transgenes that express pendrin in the presence, but not absence, of doxycycline. The transgenes were crossed onto the *Slc26a4*^{-/-} background so that all pendrin expression is derived from the responder transgene. We showed that *Slc26a4* expression from embryonic day 16.5 (E16.5) to postnatal day 2 (P2) was necessary and sufficient to acquire

normal hearing at one month of age. Lack of *Slc26a4* expression during this period led to endolymphatic acidification, reduction of the endocochlear potential (EP) and mild to severe hearing loss. Some doxycycline administration regimens caused these abnormalities without detectable scala media expansion or EVA. The timing of doxycycline administration and pendrin expression could be manipulated to generate mice with unilateral or asymmetric hearing loss associated with minimal, if any, EVA and no other morphogenetic anomalies (Choi et al., 2011). In this study, we characterized the natural history and pathogenesis of hearing loss after one month of age in Tg[E];Tg[R];*Slc26a4*^{-/-} mice which receive doxycycline from conception until E17.5 (Choi et al., 2011).

Materials and Methods

Ethics Statement

All animal experiments and procedures were performed according to protocols approved by the Animal Care and Use Committee of the National Institute of Neurological Diseases and Stroke and the National Institute on Deafness and Other Communication Disorders.

Animals

The background strain history of the effector transgene (Tg[E]; Tg(RP23-265L9/rTA2S-M2/NeoR)1A_{tg}) and responder transgene (Tg[R]; Tg(AcGFP/TRE/*Slc26a4*)2A_{tg}) lines was previously described (Choi et al., 2011). Briefly, the genetic background of each transgenic line initially included C57BL/6J and SJL, well as 129Sv/Ev derived from *Slc26a4*^{-/-} mice segregating a targeted deletion allele of *Slc26a4* (Everett et al., 2001). To induce *Slc26a4* expression, drinking water contained doxycycline hyclate (Sigma-Aldrich) and 5 g sucrose (MP Biomedicals) per 100 ml of reagent-grade water (Choi et al., 2011). Doxycycline-containing water was provided to the dam from the onset of mating and substituted with doxycycline-free water at embryonic day 17.5 as estimated from the day at which a vaginal plug was observed.

Genotype analysis

At approximately postnatal day 20 (P20), genomic DNA was prepared from tail clip specimens using the Maxwell 16 System (Promega). We performed PCR genotype analyses with Taq polymerase (GenScript) to detect the presence of the effector transgene (Tg[E]; Tg(RP23-265L9/rTA2S-M2/NeoR)1A_{tg}), the responder transgene (Tg[R]; Tg(AcGFP/TRE/*Slc26a4*)2A_{tg}) or *Slc26a4*^{-/-} as previously described (Choi et al., 2011). We defined Tg[E];Tg[R];*Slc26a4*^{-/-} mice as experimental animals (experimental DE17.5) and Tg[E];Tg[R];*Slc26a4*^{+/+} littermates or offspring of the same parents as control animals. Transgene-negative *Slc26a4*^{-/-} mice were defined as negative control animals.

Auditory brainstem response thresholds

Auditory brainstem response (ABR) thresholds were measured at 1, 2, 3, 4, 5, 6, 9, and 12 months of age in Tg[E];Tg[R];*Slc26a4*^{-/-} (experimental) and Tg[E];Tg[R];*Slc26a4*^{+/+} (positive control) mice essentially as described (Choi et al., 2011). Mice were anesthetized and placed on a heating pad. Stimuli were either 50-μs duration rarefaction clicks, or 8-, 16-, or 32-kHz tone bursts as described in Szymko-Bennett et al (Szymko-Bennett et al., 2003).

Briefly, the number of stimulus presentations was varied from 256 to 1024 depending on signal-to-noise ratio, and supra-threshold stimulus intensities were initially decreased in 10-dB steps followed by 5-dB steps at lower intensities to determine the response threshold. Some ears had no detectable waveform in response to the highest intensity level of 120 dB or 100 dB sound pressure level (SPL) for click or 32-kHz tone burst stimuli, respectively. The threshold was considered to be 125 or 105 dB SPL for subsequent analyses, respectively.

Distortion product otoacoustic emissions

Distortion product otoacoustic emissions (DPOAEs) were recorded with an acoustic probe (ER-10C; Etymotic Research, Elk Grove Village, IL) using DP2000 DPOAE measurement system version 3.0 (Starkey Laboratory, Eden Prairie, MN). Two primary tones with frequency ratio $f_2/f_1 = 1.2$ were presented at intensity levels $f_1 = 65$ dB SPL and $f_2 = 55$ dB SPL. f_2 was varied in one-sixth-octave steps from 4 to 18 kHz. Due to the limited frequency response of the acoustical transducers of the ER-10C probe, reliable DPOAE measurements were possible only at f_2 frequencies < 18 kHz. Distortion produce otoacoustic emission signal-to-noise ratio (SNR) was calculated by subtracting the noise level from the DPOAE amplitude. Negative DPOAE SNR values were considered to be zero for subsequent analyses.

Histopathology

Mice were euthanized by CO₂ exposure followed by cervical dislocation. Inner ears were dissected and processed as described (Choi et al., 2011; Noben-Trauth et al., 2010) using the JB-4 resin Kit (Polysciences Inc.) and a Leica RM2265 microtome. Sections of 4- μ m thickness were stained with 0.1% toluidine blue. We captured images with ACT-1 software and a Nikon Digital Cam DXM1200 attached to a Nikon Eclipse 90i light microscope.

Endocochlear potential

Mice were anesthetized with tribromoethanol at a dose of 0.35 mg/g body weight. Endocochlear potential measurements were made in the basal turn of the cochlea by a round-window approach through the basilar membrane of the first turn (Wangemann et al., 2004; Wangemann et al., 2007). Glass microelectrodes were prepared as described (Choi et al., 2011; Noben-Trauth et al., 2010). Anoxia was induced by intramuscular injection of succinylcholine chloride (0.1 μ g/g) after establishment of deep anesthesia followed by additional injection of tribromoethanol. Data were recorded digitally (Digidata 1440A and AxoScope 10; Axon Instruments) and analyzed using Clampfit10.

Immunohistochemistry

Whole-mounted or sectioned mouse cochleae were immunostained essentially as described for KCNJ10, KCNQ1, SLC12A2 (Wangemann et al., 2004), Prestin (Belyantseva et al., 2000), CD34 (Hertzano et al., 2011) or CD68 (Jabba et al., 2006) with some modifications: the blocking solution was PBS with 5% bovine serum albumin. Primary antibodies were diluted 1:300 (rabbit anti-KCNJ10; Alomone, Jerusalem, Israel), 1:200 (goat anti-KCNQ1; Santa Cruz Biotechnology, Santa Cruz, CA), 1:100 (goat anti-SLC12A2; Santa Cruz

Biotechnology), or 1:200 (goat anti-prestin, Santa Cruz Biotechnology) in blocking solution. The secondary antibody was FITC-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology) or Alexa Fluor 568-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA) diluted 1:500. Strial blood vessels and macrophages were labeled with Alexa Fluor 647-conjugated rat anti-CD34 (BD Biosciences Pharmingen, San Diego, CA) and Alexa Fluor 488-conjugated rat anti-CD68 (AbD Serotec, Raleigh, NC), respectively, at 1:100 dilution. Tissues were counterstained with phalloidin (Molecular Probes, Eugene, OR) at 1:100 dilution. Slides were mounted with ProLong Gold antifade reagent (Invitrogen). Images were captured with an LSM 780 confocal microscope equipped with ZEN 2010 software (Zeiss). Quantitation of anti-CD68 staining area was performed equivalently for all images using the area calculation function of ImageJ (<http://rsbweb.nih.gov/ij/>).

AM1-43 uptake

Organs of Corti were excised from 1 month-old experimental or control mice. N-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl)pyridinium dibromide (AM1-43, Invitrogen) was applied at 5 μ M to the mounted tissue for 1 min as previously described with some modifications (Meyers et al., 2003; Taylor et al., 2008). Just after application of AM1-43, the tissue was immediately fixed by local perfusion of 4% PFA through the oval and round windows to inhibit endocytic uptake of AM1-43. Images were obtained with an LSM 780 confocal microscope (Zeiss).

Tracer studies

Vascular permeability was assessed using various tracers, including Evans blue (molecular mass = 961 Da) (Sigma Aldrich, St Louis, MO), lysine-fixable cadaverine conjugated to Alexa Fluor-555 (molecular mass = 950 Da) (Invitrogen), or FluoSpheres®, fluorescent microspheres with diameters 0.02, 0.2 or 1.0 μ m (Molecular Probes). After the mice were deeply anesthetized, a 30-Ga blunt-ended needle was used to inject tracer solution through the left ventricle for systemic intravascular perfusion with a low-pressure pump. The mice were decapitated, and their cochleae were harvested immediately. Whole-mounted cochlear lateral wall tissue was dissected carefully for each tracer perfusion. Tracer in the lateral wall was visualized using a fluorescent microscope. Strial tight junction barrier integrity was tested as previously described (Gow et al., 2004; Kitajiri et al., 2004). Briefly, perilymphatic injection and incubation was performed with 100–200 μ l EZ-Link Sulfo-NHS-LC-Biotin (Pierce Chemical, Rockford, IL) at 10 mg/ml in PBS containing 1 mM CaCl_2 for 5 minutes followed by 5 serial perfusions with 1 ml PBS containing 1 mM CaCl_2 to wash out residual biotin. The temporal bones were then fixed by perilymphatic perfusion with 4% PFA for 2 hours and processed for immunofluorescence microscopy. The distribution of biotin tracer was visualized by incubating frozen sections with streptavidin-FITC conjugate (Invitrogen) at 1:500 dilution. Biotin was confirmed to reach the scala media by its staining of the tectorial membrane, the apical surface of marginal cells, and fibrocytes underlying the stria vascularis.

Quantitative RT-PCR

At least 5 cochlear lateral walls were excised from each genotype group at 1 and 3 months of age. RNA was extracted and quality was confirmed using an Agilent Bioanalyzer

(Agilent Technologies). RNA was reverse transcribed into cDNA for qPCR analysis with efficient primer sets specific to *Actb* or *Cd68* with ZEN double-quenched probes containing a 5' FAM fluorophore, 3' IBFQ quencher, and an internal ZEN quencher (IDT, Coralville, IA). Sequences for primers and probes are: 5'-AGGTCTTTACGGATGTCAACG-3', 5'-TCACTATTGGCAACGAGCG-3' and 5'-AAAAGAGCC/ZEN/TCAGGGCATCGGAA-3 for *Actb*; 5'-GTGTCTGATCTTGCTAGGACC-3', 5'-TGTGCTTTCTGTGGCTGTAG-3' and 5'-TGAAGGATG/ZEN/GCAGGAGAGTAACGG-3 for *Cd68*. Comparative TaqMan assays were performed on an ABI 7500 real-time PCR system (Applied Biosystems). PCR reactions were performed in a 10- μ l volume containing 2 μ l cDNA, 1 μ l primer mix (IDT), 5 μ l of Universal PCR Master Mix (Applied Biosystems) and 2 μ l water. Cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Relative expression was normalized to the level of β -actin expression (encoded by *Actb*), and calculated as 2^{-CT} using the comparative threshold cycle method (Kurima et al., 2011). Data are presented as mean values $\pm$ S.E. of 3 technical replicates for each genotype. One-way ANOVA was used to identify statistically significant differences between genotype groups at each time point or between different time points for each genotype.

Statistics

Statistical analyses included Pearson correlation, Fisher's exact test, unpaired Student's *t* test and one-way ANOVA. $p < 0.05$ was considered to be significant. Pearson correlations were described as strong if $-1.0 < R < -0.5$ or $0.5 < R < 1.0$, moderate if $-0.5 < R < -0.3$ or $0.3 < R < 0.5$, or weak if $-0.3 < R < -0.1$ or $0.1 < R < 0.3$.

Results

Auditory brainstem response thresholds

We sought to characterize the natural history hearing loss in Tg[E];Tg[R];*Slc26a4*^{+/−} mice in which *Slc26a4* expression was driven by doxycycline administration from conception to embryonic day 17.5 (E17.5). These mice were previously reported to have partial hearing loss at 1 month of age (Choi et al., 2011). We hypothesized that their hearing would fluctuate or progress as it does in many patients with Pendred syndrome. We measured auditory brainstem response (ABR) thresholds for each ear of experimental (Tg[E];Tg[R];*Slc26a4*^{+/−}) and positive control (Tg[E];Tg[R];*Slc26a4*^{+/+}) mice, all exposed to doxycycline from conception to E17.5, at 1, 2, 3, 4, 5, 6, 9 and 12 months of age. Positive control ears that express *Slc26a4* from one endogenous *Slc26a4* wild type allele had stable mean click-stimulus thresholds from 1 through 12 months (Fig. 2A). The experimental ears, in which all *Slc26a4* was expressed from the responder transgene under doxycycline-inducible control, had similar mean click-stimulus thresholds from 1 through 6 months, followed by a significant ($p < 0.0001$) elevation of mean thresholds indicative of hearing loss progression at 9 and 12 months of age (Fig. 2B). The ABR threshold results for 8-kHz, 16-kHz and 32-kHz tone burst stimuli were similar to those for click stimuli (Fig. S1A,B). We then calculated the prevalence of hearing loss progression. More than 60% of all experimental ears showed progression of hearing loss defined as a >20 dB increase in thresholds for an ear between 1 month of age and the final time point of measurement, either

9 or 12 months of age (Fig. S1C). If this analysis only included ears that initially had normal hearing or less severe hearing loss (ABR threshold <50 dB SPL at 1 month of age), hearing loss progression was observed in 75% of ears (Fig. S1C).

During the first six months of age, many experimental mice had ABR thresholds that increased, decreased, or both (fluctuated) in one or both ears. An example of an ear with severe hearing loss fluctuation is shown in Fig. S2. To measure this fluctuating hearing loss, we calculated ABR threshold differences in individual ears at 1-month intervals of age (Fig. 2C). Control ears rarely showed threshold differences greater than 10 dB, while experimental ears revealed a wide range of threshold differences. If fluctuating hearing loss was defined as a >10-dB upward or downward threshold difference for a 1-month age interval (Fig. 2D), 57.6% of experimental ears had hearing loss fluctuation between 1 and 2 months of age or between 2 and 3 months of age. Finally, we sought to identify differences in fluctuation of ABR thresholds in response to different pure-tone frequency stimuli. The total fluctuation of hearing at each ABR stimulus frequency was calculated as the cumulative total of the absolute value of each ABR threshold difference for all 5 one-month age intervals, up to 6 months of age, for each ear. The mean cumulative fluctuation was calculated separately for each genotype for each tone-burst stimulus: 8, 16 and 32 kHz (Fig. 2E). While the mean cumulative fluctuation in experimental ears showed significantly larger threshold differences ($p < 0.0001$) compared to those of control ears for each stimulus frequency, there was no difference ($p = 0.27$) among mean cumulative fluctuation values among different stimulus frequencies by one-way ANOVA testing.

Endocochlear Potential

We hypothesized that fluctuating hearing loss was caused by fluctuation of the EP. The mean EP in control ears at 1 and 3 months of age was 98.1 mV ($n = 6$) and 99.4 mV ($n = 12$), respectively (Fig. 3A), similar to previously reported values (Royaux et al., 2003). In comparison to control mean EP values, the mean EP in experimental ears was significantly decreased to 63.4 mV ($n = 15$, $p = 0.0003$) and 63.6 mV ($n = 18$, $p < 0.0001$), respectively (Fig. 3A).

Next, we sought to determine if EP values were correlated with ABR thresholds. Because our EP measurement protocol was a non-survival procedure, it was not possible to longitudinally follow the EP in individual ears of any one mouse; therefore we performed a cross-sectional correlative analysis of EP values with ABR thresholds in experimental ears at 1 and 3 months of age (Fig. 3B). There were strong negative correlations of EP values with click ABR thresholds at 1 month ($R = -0.79$, $p < 0.001$) and 3 months of age ($R = -0.74$, $p < 0.001$). These results implicate fluctuation of the EP as the cause of fluctuation of hearing in DE17.5 experimental mice between 1 and 3 months of age.

Hair cell function and structure

We also measured the EP under anoxic conditions as an indirect indicator of hair cell function. In a normal mouse, a few minutes of anoxia interferes with active transport in the stria vascularis and causes the EP to decline from a normal positive potential of 80 to 100 mV to approximately -30 to -40 mV (Dallos, 1983; Johnstone, 1965; Royaux et al., 2003;

Sadanaga and Morimitsu, 1995). This negative value is thought to reflect persistent activity of viable hair cells (Dallos, 1983). The mean anoxic EP of experimental ears was -25.9 mV ($n = 7$), which was not statistically different ($p = 0.15$) from the mean value of -30.8 mV in anoxic control ears ($n = 8$) at 3 months of age (Fig. 3C). This result suggests that hair cell function was not severely impaired in experimental ears up to 3 months of age.

Measurement of distortion product otoacoustic emissions (DPOAE) is a non-invasive method to assess the function of outer hair cells (Brownell, 1990; Kemp, 1978; Noguchi et al., 2006). We asked whether decreased EP, which reduces the electrochemical driving force for cation entry into hair cells, resulted in reduced DPOAE amplitudes in experimental ears. Fig. S3A shows mean DPOAE signal to noise ratios (SNRs) for control and experimental ears at the approximate age of 1 month. Distortion product otoacoustic emissions were absent in both control and experimental ears when the stimulus $f_2 = 4$ kHz, but control DPOAE SNRs rose sharply in response to stimuli at f_2 frequencies above 4 kHz. The mean DPOAE SNRs of experimental ears exhibited significant reductions in comparison to those of control ears at all f_2 stimulus frequencies except 4 kHz (Fig. S3A). We then tested the hypothesis that DPOAE SNRs were correlated with ABR thresholds for individual ears at stimulus frequencies of 4 and 8 kHz. Distortion product otoacoustic emission SNRs at stimulus frequency $f_2 = 7.9$ kHz were strongly correlated ($R = 0.62$, $p < 0.0001$; Fig. S3B) with ABR thresholds for 8-kHz tone-burst stimuli and, similarly, DPOAE SNRs at $f_2 = 15.9$ kHz were strongly correlated ($R = 0.70$, $p < 0.0001$; Fig. S3C) with ABR thresholds for 16-kHz tone-burst stimuli. These data indicate that outer hair cell function is impaired in experimental ears. However, the presence of a normal anoxic EP in experimental ears suggests that the impairment of outer hair cell function is not caused by a primary defect of hair cells, but by an indirect effect of reduction of the EP.

To characterize the cause of decreased DPOAE amplitudes in experimental ears, we examined the expression of prestin. Prestin is a motor protein expressed in outer hair cells that is required for the generation of DPOAEs (Belyantseva et al., 2000; Liberman et al., 2002; Zheng et al., 2000). Prestin expression and localization appeared normal in control and experimental ears at 3 months of age (Fig. S4). This result indicates that loss of the EP, but not loss of the outer hair cells, accounts for the loss of outer hair cell function in experimental ears. To further assess the functional status of hair cells in experimental ears with severe hearing loss, we evaluated the hair cell uptake of the styryl membrane dye AM1-43, a fixable analog of FM1-43, which is thought to reflect the presence of functional mechanotransduction channels open at rest (Meyers et al., 2003; Taylor et al., 2008). Hair cells in control and experimental ears showed similar specific uptake following a brief exposure to AM1-43 (Fig. S4). Taken together, our results indicate that experimental hair cells are functionally and morphologically intact and are therefore not the underlying cause of hearing loss in experimental ears.

Cochlear duct histomorphology

To identify histopathologic changes that could account for the observed physiological and functional abnormalities, we used light microscopy to quantitatively evaluate histologic cross sections of cochleae at 3 months of age (Fig. 4). The mean thickness of stria vascularis

in experimental ears was significantly greater than that of control ears at apical, middle and basal turns ($p < 0.005$, $p < 0.005$, and $p < 0.05$, respectively). Other structural features of experimental cochlear ducts did not appear different from those of control ears (Fig. 4). We could not identify structural abnormalities of the organ of Corti, spiral ganglion, or other regions of the lateral wall to account for the hearing loss. We observed distention of Reissner's membrane, indicative of endolymphatic hydrops, in 1 of 6 experimental ears at 1 month of age and zero of 8 experimental ears at 3 months of age.

Stria vascularis barrier integrity

We hypothesized that the decreased EP in experimental (Tg[E];Tg[R];*Slc26a4*^{-/-}) mice could be due to disruption of the tight junction barrier of the basal or marginal cell layers of the stria vascularis (Gow et al., 2004; Kitajiri et al., 2004). We tested this hypothesis by perilymphatic perfusion of biotin in 1-month-old ears (Gow et al., 2004; Kitajiri et al., 2004). Biotin was excluded from the intrastrial space in both positive control (Tg[E];Tg[R];*Slc26a4*^{+/+}) and experimental ears (Fig. 5A,B). In contrast, some biotin was detectable in the stria vascularis of negative control (*Slc26a4*^{-/-}) ears (Fig. 5C). We also assessed the integrity of the blood-labyrinth barrier within the stria vascularis through systemic intravascular injections of Evans blue, or small-, medium- or large-mass fluorescent tracers (0.02, 0.2 and 1.0 μ m, respectively). The tracers did not accumulate in the intrastrial fluid space of either experimental ears or positive or negative control ears (Fig. 5D–L). We did observe truncation of blood vessels in the stria vascularis of negative control (*Slc26a4*^{-/-}) ears (Fig. 5F,I,L). Taken together, these results indicate that the reduction of the EP in the ears of experimental mice is not due to a disruption of the vascular endothelial cell barrier or marginal or basal cell tight junction barriers in the stria vascularis.

Stria vascularis morphology and gene expression

Since the EP is generated by the stria vascularis, we examined morphology and protein marker expression of the stria vascularis at 1 month of age. KCNQ1 expression on the apical surface of marginal cells (MCs), facing the endolymph, was indistinguishable between positive control (Fig. 6A,B) and experimental ears (Fig. 6C,D). We used anti-KCNJ10 antibodies to label intermediate cells, which showed lower overall levels of KCNJ10 in experimental ears (Fig. 6H,I,J) in comparison to positive control ears (Fig. 6E,F,G) at 1 month of age. Furthermore, the intermediate cells in experimental ears (Fig. 6I,J) lost much of their elaborate network of fine convoluted extensions oriented toward the basal cell layer in positive control ears (Fig. 6G,H). These extensions interdigitate with a similar network of extensions of the basolateral membrane of marginal cells that we visualized by labeling with anti-SLC12A2 (NKCC1) antibodies (Fig. 6K,L,M,N). The results confirmed a loss of this extensive morphological specialization of the intermediate cell layer in experimental ears (Fig. 6N). These changes in intermediate cell morphology and expression of KCNJ10, required for generation of the EP itself across the intermediate cell layer, may account for the reduction of the EP in experimental ears.

Macrophages in the stria vascularis

We used anti-CD68 antibodies to label macrophages and anti-CD34 antibodies to label blood vessels in the stria vascularis of positive control, experimental, and negative control mice at 1 month of age (Fig. 7). Anti-CD68 staining was observed adjacent to the abluminal surfaces of blood vessels in all three groups (Fig. 7A–C). The amount (area) of staining was significantly increased ($p < 0.001$) in experimental and negative control ears compared to those of positive control ears (Fig. 7D). Anti-CD68 staining area was measured for each ear and strongly correlated ($R = 0.64$, $p < 0.005$) with the click ABR threshold in the corresponding ear in experimental animals (Fig. 7E). A strong correlation with click ABR thresholds was also observed by quantitative real-time PCR analysis of *Cd68* mRNA levels at 1 month ($R = 0.79$, $p < 0.05$). The mean level of *Cd68* mRNA in experimental ears was significantly higher ($p < 0.05$) compared to that in positive control ears, but a positive correlation was not seen at 3 months of age ($R = -0.39$) (Fig. 8).

Pigment granule accumulation

We explored the relationship between CD68 staining, pigment granules and KCNJ10 expression in experimental ears. Fig. 9 (B,D) shows a representative experimental stria vascularis with larger pigment aggregates, but not smaller pigment granules, associated with CD68 staining at 1 month of age. In contrast, all or nearly all of the visible pigmentation appeared to be in direct apposition or juxtaposition with KCNJ10 (Fig. 9E–G), suggesting that pigmentation arises in intermediate cells. We rarely observed pigmentation in positive control (Tg[E];Tg[R];*Slc26a4*^{+/+}) ears at 1 month of age (Fig. 9A,C), indicating that increased pigment accumulation specifically occurs in experimental ears, likely as a result of oxidative stress (Singh and Wangemann, 2008).

Discussion

Fluctuations and sudden drops of hearing are the most distinctive and enigmatic features of hearing loss caused by mutations of *SLC26A4* (Griffith and Wangemann, 2011; King et al., 2010). The etiology and pathogenesis of hearing loss fluctuation in this disorder were previously unknown due, in large part, to the lack of an animal model with this phenotype. We previously engineered a binary transgenic mouse line with doxycycline-inducible expression of *Slc26a4* and showed that *Slc26a4* expression is required only during a critical interval from embryonic day 16.5 (E16.5) to postnatal day 2 (P2) for acquisition of normal auditory brainstem response (ABR) thresholds at 1 month of age (Choi et al., 2011). Here we used this transgenic line to generate *Slc26a4*-insufficient mice by administering doxycycline from conception to E17.5 (termed DE17.5). If the half-life of induced SLC26A4 protein (pendrin) were estimated to be 18 hours (Choi et al., 2011), the amount of pendrin at the end of the critical interval (P2) would be approximately 1% of the amount at E17.5, when doxycycline administration was discontinued. The resulting *Slc26a4*-insufficient mouse model has hearing loss fluctuation and progression that approximates what is observed in many patients with enlargement of the vestibular aqueduct, either in isolation or in association with Pendred syndrome (Choi et al., 2011). We have probably underestimated the degree and prevalence of fluctuation due to the inability to mask stimulation of the contralateral ear in unilateral or asymmetric hearing loss in mice, as well

as the likelihood that fluctuation also occurred between measurement time points. We conclude that *Slc26a4* expression is required for the development of stable hearing, maintenance of stable hearing in an ear whose development has been disrupted by a loss or reduction of *Slc26a4*, or both of these mechanisms. The fluctuating and progressive hearing loss approximating the human phenotype indicates the DE17.5 paradigm is a useful and unique animal model to study hearing loss caused by *SLC26A4* mutations.

The etiology and pathogenesis of fluctuating sensorineural hearing loss in humans have long remained obscure. Another well-described but idiopathic disorder with hearing loss fluctuation is Meniere disease, in which endolymphatic hydrops was historically thought to underlie hearing loss (Schuknecht et al., 2010). There is a guinea pig model with fluctuating hearing loss (Kimura, 1967), and only one genetic mouse model that we are aware of: the *Phex* mutant mouse (Megerian et al., 2008). The mechanism of fluctuation in *Phex* mutants is not clear but appears to include spiral ganglion dysfunction and endolymphatic hydrops. The phenotype of the *Phex* mouse and our experimental DE17.5 phenotype indicate the existence of multiple pathogenetic pathways for hearing loss fluctuation, and emphasize the need for additional models.

Fluctuation of hearing loss in experimental DE17.5 mice was not associated with detectable defects of hair cells or the spiral ganglion, endolymphatic hydrops, alterations of stria vascular permeability or tight junction barriers, or osseous malformations (Fig. 3C, 4, 5, Fig. S3, 4). We cannot entirely rule out undetected mild EVA itself as a partial or indirect contributor to hearing loss. However, the recapitulation of a highly penetrant hearing loss phenotype in so many ears without detectable EVA suggests that structural malformations are not a major contributor. Rather, we conclude that the direct cause of hearing loss fluctuation is a cyclical pathologic process directly affecting the intermediate layer of the stria vascularis and the generation of the endocochlear potential.

The intermediate cells are irregular in shape and have an extensive and convoluted network of cytoplasmic processes that protrude toward and interdigitate with a similar network of processes extending from the marginal cells (Fig. 6) (Mizuta et al., 1997; Spicer and Schulte, 2005; Takeuchi et al., 2001). Our confocal microscopic studies of immunostained cell layers of the stria vascularis demonstrated the degeneration of this network of interdigitations of the intermediate and marginal cells in our experimental DE17.5 mice (Fig. 6L,N). Moreover, the intermediate cell surfaces apposing the basal cell layer were abnormal (Fig. 6F,G,I,J), with a reduction of plasma membrane surface area and a likelihood of impaired intercellular communication with basal cells that could reduce K⁺ uptake into intermediate cells. This could contribute to a loss or instability of the endocochlear potential. We conclude these disruptions of intermediate layer morphology, in combination with loss of expression of essential proteins such as KCNJ10, are the direct cause of the loss of the endocochlear potential and hearing in experimental DE17.5 mice. This otopathologic mechanism could also underlie the loss or fluctuation of the endocochlear potential and hearing in other disorders. However, although intermediate cells seem to be irreversibly damaged and the primary anatomic site of lesion, we cannot rule out secondary degeneration due to physiologic dysfunction of marginal cells or other cells of the stria vascularis or inner ear.

Based upon our current results and previously published data, we hypothesize the following model for the pathogenesis of hearing loss in experimental DE17.5 ears up to 3 months of age: (1) loss or reduction of *Slc26a4* expression after E17.5; (2) acidification of endolymph; (3) acquisition of normal or near-normal hearing at 3–4 weeks of age; and (4) a pathologic cyclical process involving oxidative stress (Singh and Wangemann, 2008) and loss of KCNJ10, the endocochlear potential, and hearing. The roles of hyperpigmentation, macrophage invasion, activation or proliferation in the intermediate layer, and macrophage phagocytosis remain unclear. The histologic hallmark of this pathogenic process is degeneration of the intermediate layer of the stria vascularis.

It is instructive to note the similarity of the blood-labyrinth barrier to the blood-brain barrier since there is an extensive literature on the latter in both health and disease (Abbott et al., 2006; Janzer and Raff, 1987; Juhn and Rybak, 1981; Zhang et al., 2012). For example, the stria vascularis CD68-positive cells that we and other authors have described are probably resident macrophages analogous to the microglia of the central nervous system (Zhang et al., 2012). Similarly, the intermediate cells of the stria vascularis show embryologic, morphologic and functional similarities to astrocytes (Shi, 2011; Takeuchi et al., 2001). Finally, there is the strial vasculature itself, which could underlie the instability of hearing in experimental DE17.5 ears if sudden hearing loss is due to microvascular occlusion or alterations. Although we could not find evidence for abnormal permeability of the strial vasculature, it remains possible that our methods failed to detect physiologically relevant alterations of vascular permeability, which are thought to affect the endocochlear potential. Our methods may also have failed to detect pathologic alterations of the permeability of other tight junction barriers such as the marginal and basal layer barriers. It is also possible that the morphologically observable alterations associated with sudden hearing loss are temporary and we simply did not detect them due to the prospective temporal schedule of our evaluations. Histopathologic examinations immediately following a sudden hearing loss could be illustrative, but are limited by the frequency with which hearing can be tested in animals. This limitation arises out of the need for anesthesia to test monaural ABR thresholds in our model since behavioral response testing (such as the Preyer reflex) will not detect partial or unilateral hearing loss.

One of the most distinctive and enigmatic features of hearing loss in some patients with Pendred syndrome, and EVA, in general, is sudden hearing loss caused by minor head trauma or barotrauma (Griffith and Wangemann, 2011; Jackler and De La Cruz, 1989). Traumatic brain injury is known to be associated with microvascular leakage, oxidative and free radical stress, microglial activation, and alterations of astrocyte function (Bruce et al., 1996; Globus et al., 1995; Horner and Gage, 2000). Perhaps hearing loss in EVA is caused by minor head trauma that occurs via similar mechanisms affecting a blood-labyrinth barrier that is structurally or functionally compromised by disruption of strial development and homeostasis. Future studies are needed to test this hypothesis.

Our experimental DE17.5 mouse paradigm now provides a model to rigorously test potential therapeutic or preventive interventions for hearing loss associated with Pendred syndrome and EVA. The pathologic process involving the intermediate layer of the stria vascularis in experimental DE17.5 mice may be a final common pathway to the loss of EP and hearing

elicited by a variety of etiologies. Replacement or regeneration of intermediate cells could be a strategic approach to treatment of Pendred syndrome, EVA, as well as other disorders acting via this pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Schematic illustrations of mammalian inner ear anatomy. **(A)** The inner ear consists of the cochlea, the auditory organ, and the vestibular end organs. The utricle and saccule detect linear acceleration, and the semicircular canal ampullae detect angular acceleration. The membranous labyrinth (shown) is encapsulated in bone (not shown). **(B)** The cochlear duct includes the scala media comprised of endolymph. Other parts of the cochlea, including the scalae vestibuli and tympani, are comprised of perilymph. The endolymph is separated from perilymph by the stria vascularis (SV), spiral ligament (SL), organ of Corti (OC) and Reissner's membrane (RM). The stria vascularis generates the endocochlear potential largely by secretion of K^+ into endolymph. The organ of Corti contains the sensory hair cells. Pendrin is expressed in epithelial cells of the spiral prominence (SP), root cells (R), and spindle cells of the stria vascularis. **(C)** The stria vascularis consists of three cell layers: marginal cell (MC), intermediate cell (IC) and basal cell (BC) layers. The stria vascularis is electrochemically isolated from endolymph and perilymph by tight junctions among marginal cells and basal cells, respectively. Tight junctions in the vasculature also contribute to an electrochemical blood-labyrinth barrier. KCNJ10 (Kir 4.1) channels (red) are located in the highly convoluted plasma membrane of intermediate cells facing the intrastrial space. The sodium-potassium co-transporter SLC12A2 (NKCC1) (green) is located in the basolateral membrane of marginal cells, while the KCNQ1 (Kvlqt1)/KCNE1 (Isk) potassium channel is located in the apical membrane of marginal cells. Reprinted with permission from the JCI (Choi et al., 2011) and Physiological Society (Wangemann, 2006).

Figure 2.

Auditory brainstem response (ABR) thresholds. (**A, B**) Mean ($\pm$ S.D.) click stimulus-elicited ABR thresholds for all ears of control (**A**) or experimental mice (**B**). The number of tested ears is shown above each bar. The difference between control and experimental means for each time point was significant (one-way ANOVA, $p < 0.0001$). Asterisks indicate experimental mean values at 9 and 12 months of age were significantly greater (Tukey post hoc test, $p < 0.05$) than those at earlier time points. (**C**) Click ABR threshold shifts in individual ears at the indicated 1-month intervals of age. Threshold elevations (hearing loss) are shown as positive shifts and decreases (hearing improvement) are displayed as negative shifts. Inter-test variation up to 10 dB is clinically defined as normal so threshold shifts >10 dB were considered significant. (**D**) Prevalence of click ABR threshold shifts >10 dB for individual ears measured at the indicated age intervals. 57.6% of experimental ears showed a significant threshold shift during the 1–2 month or 2–3 month age interval. Asterisks indicate significant differences between control and experimental ears at 1–2 month, 2–3 month or 3–4 month age intervals (Fisher's exact test, $p = 0.0004$, $p = 0.0001$ or $p = 0.0041$, respectively). (**E**) Mean ($\pm$ S.D.) total fluctuation for individual ears, measured as the mean of the cumulative total of absolute values of ABR threshold shifts. Asterisks indicate significant differences between the mean total fluctuation in control versus experimental ears by click, 8-, 16- or 32-kHz stimulus (unpaired t test, $p < 0.0001$, $p < 0.0001$, $p = 0.0001$, or $p < 0.0001$, respectively). In contrast, there was no difference among mean total fluctuation values for different stimulus frequencies among experimental ears (one-way ANOVA, $p = 0.27$).

Figure 3.

Endocochlear Potential (EP). **(A)** Mean EP ($\pm$ S.E.) values measured at 3 months of age. The mean EP for experimental ears was significantly lower (unpaired t test, $p < 0.0001$) than the value for control ears. **(B)** Correlation between individual ear EP measurements and click ABR thresholds at 1 and 3 months of age ($R = -0.79$, $p = 0.0005$, or $R = -0.74$, $p = 0.0005$, respectively). The mean ($\pm$ S.D.) of positive control EP values is shaded. **(C)** Mean EP ($\pm$ S.E.) values measured at 3 months of age under anoxic conditions. There was no significant difference (unpaired t test, $p = 0.15$) between experimental and control EP values.

Figure 4.

Cochlear duct histomorphology. Light microscopic images of positive control (A) and experimental (B) mice at 3 months of age. RM; Reissner membrane, SV; stria vascularis, SLm, spiral limbus; TM, tectorial membrane; SLg, spiral ligament; OC, organ of Corti, SG; spiral ganglion. The stria vascularis of experimental mice is bulky and vacuolated. The spiral ganglion, organ of Corti, Reissner membrane and other structures of experimental mice look similar to those of positive control mice.

Mean ($\pm$ S.E.) stria thickness (C), spiral ganglion cell density (D) and outer hair cell survival rate (E). $n = 8$ ears for each genotype (C,D,E). Inner hair cell survival rate was 100% for all genotypes. The only observed difference between control and experimental ears is the stria thickness, suggesting that the stria vascularis of experimental ears is affected by local edema and, probably, inflammation. Asterisks indicate significant differences: ** $p = 0.0033$, *** $p = 0.0005$, and * $p = 0.023$.

Figure 5.

Barrier integrity of the stria vascularis. (A,B,C) Representative cryosection images of the stria vascularis, outlined by dotted white lines, of positive control (A), experimental (B) and negative control (C) ears after perilymphatic infusion of biotin. There is slight penetration of biotin into the stria vascularis of negative control mice but not of positive control or experimental mice.

Scale bar: 50 μm (A, applies to B and C). (D–L) Representative confocal microscopic images of positive control (D,G,J), experimental (E,H,K), or negative control (F,I,L; *Slc26a4*^{-/-}) stria vascularis labeled with anti-CD34 antibodies following cardiac perfusion of 1- μm (D,E,F), 0.2- μm (G,H,I) or 0.02- μm intravenous tracers (J,K,L). The blood-labyrinth barrier appears to be functionally intact in the stria vascularis from mice of all genotypes. Results were confirmed with at least 3 ears of each genotype with each of the tracers. Scale bar: 40 μm (D, applies to E–L). Truncated blood vessels are indicated (★).

Figure 6.

Intermediate layer morphology and function. (A–D) Anti-KCNQ1 staining of the apical plasma membrane of marginal cells of the stria vascularis of positive control (A,B) and experimental mice (C,D). Scale bars: 50 μ m (A, applies to C), 20 μ m (B, applies to D). There was no detectable difference in anti-KCNQ1 staining levels or patterns between experimental and control mice. (E–J) Anti-KCNJ10 staining of the plasma membrane of intermediate cells of positive control (E,F,G) and experimental mice (H,I,J) shown in cryosections (E,H), confocal surface images of whole-mounted tissue (F,I) and corresponding Z-stack reconstructions (G,J). There is a reduction of anti-KCNJ10 staining and intermediate cell plasma membrane extensions in experimental ears. Scale bars: 100 μ m (E, applies to H), 20 μ m (F, applies to I), 5 μ m (G, applies to J). (K–N) Anti-SLC12A2 (green) and anti-KCNJ10 (red) labeling of positive control (K,L) or experimental stria vascularis (M,N). Experimental mice show a reduction of the network of interdigitated network of extensions of marginal cells with intermediate cells observed in positive control mice. Scale bars: 20 μ m (K, applies to M), 5 μ m (L, applies to N). BC, basal cell; IC, intermediate cell; CV, capillary vessel. Bright-field image corresponding to I is provided in Fig. S5.

Figure 7.

Macrophages in the stria vascularis. (A,B,C) Anti-CD68 antibodies (green) stain cells of the macrophage-monocyte lineage in positive control (A), experimental (B) and negative control (C) stria vascularis at 1 month of age. All samples were harvested from the same corresponding region in the middle turn of the cochlear duct. Blood vessels were stained with anti-CD34 antibodies (blue). Scale bar: 40 μ m (A, applies to B and C). (D) The mean areas of anti-CD68 staining among each genotype were significantly different (one-way ANOVA, $p < 0.0001$). The mean area of anti-CD68 staining was higher in experimental ears compared to positive control ears (Tukey post hoc test, $p < 0.001$), and the mean area of staining was even higher in negative control ears (Tukey post hoc test, $p < 0.001$). (E) The area of anti-CD68 staining is correlated ($R = 0.64$, $p = 0.0029$) with click ABR thresholds in ears of experimental mice.

Figure 8.

Macrophage marker RNA expression levels in the stria vascularis measured by quantitative RT-PCR. (A,C) Mean ($\pm$ S.E.) mRNA level of *Cd68* at 1 (A) and 3 (C) months of age. One-way ANOVA was used to compare mean values among positive control, experimental and negative control ears. After ANOVA testing showed a significant difference at 1 and 3 months of age ($p = 0.0002$ for each time point), the Tukey test for pair-wise comparison was performed. Asterisks indicate significant differences: ** $p < 0.001$, * $p < 0.05$. (B,D) Correlation of mean ($\pm$ S.E.) mRNA level of *Cd68* with click ABR thresholds at 1 (B) and 3 (D) months of age. *Cd68* mRNA levels at 1 month of age were strongly correlated ($R = 0.79$, $p = 0.02$) with click ABR thresholds in ears of experimental mice. No correlation of *Cd68* mRNA level with click ABR thresholds was seen in experimental ears at 3 months of age ($R = -0.39$, $p = 0.34$).

Figure 9.

Pigmentation, macrophages and intermediate cells. (A–D) Pigment granules were rarely seen in positive control stria vascularis (A,C), while large aggregates of pigment localized with or adjacent to CD68 staining in experimental stria vascularis at 1 month of age (B,D). In contrast, small pigment granules were not associated with CD68 staining (B), suggesting that pigment granules do not initially form in macrophages. Macrophages were labeled by anti-CD68 antibodies (green). Arrows indicate large aggregates of pigment. Scale bar: 50 μ m (C, applies to A–D). (E–G) Representative merged images showing that pigment is nearly always juxtaposed with or adjacent to KCNJ10 staining at 3 months of age (E,G), suggesting that pigment is initially formed in intermediate cells. Pigment granules were generally larger in older ears. Intermediate cells were labeled with anti-KCNJ10 antibodies (red) and nuclei were labeled by DAPI (blue). (C,D,F) Pigment visualized by differential interference contrast (DIC). Scale bar: 20 μ m (E, applies to F and G).