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Outer hair cell–specific prestin-CreER^{T2} knockin mouse lines

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Abstract

Outer hair cells (OHCs) in the cochlea are crucial for the remarkable hearing sensitivity and frequency tuning. To understand OHC physiology and pathology, it is imperative to use mouse genetic tools to manipulate gene expression specifically in OHCs. Here, we generated 2 prestin knockin mouse lines: 1) the prestin-CreER^{T2} line, with an internal ribosome entry site (IRES)-CreER^{T2}-FRT-Neo-FRT cassette inserted into the prestin locus after the stop codon, and 2) the prestin-CreER^{T2}-NN line, with the FRT-Neo-FRT removed subsequently. We characterized the inducible Cre activity of both lines by crossing them with the reporter lines CAG-eGFP and Ai6. Cre activity was induced with tamoxifen at various postnatal ages and only detected in OHCs, resembling the endogenous prestin expression pattern. Moreover, prestin-CreER^{T2} +/− (heterozygotes) and +/+ (homozygotes) as well as prestin-CreER^{T2}-NN +/− mice displayed normal hearing. These 2 prestin-CreER^{T2} mouse lines are therefore useful tools to analyze gene function in OHCs *in vivo*.

Keywords

Prestin; Cre recombinase; inner ear; outer hair cells

Introduction

Postnatal cochlear outer hair cells express high level of prestin, a membrane motor protein that undergoes voltage-dependent conformational changes and consequently cell length changes or electromotility (Zheng *et al.*, 2000). Prestin-mediated OHC electromotility is necessary for cochlear amplification that is critical for remarkable hearing sensitivity and frequency tuning (Dallos *et al.*, 2008; Liberman *et al.*, 2002). In addition, prestin mRNA, not protein, is also detected at low levels in brain, testis and vestibular hair cell (Adler *et al.*, 2003; Zheng *et al.*, 2003). However, the function of many other proteins in OHC cytoskeleton regulation, synaptic formation and stereociliary mechanics, is still poorly understood, because of the early embryonic lethality or the complex pleiotropic effects associated with loss-of-function mutations in the corresponding genes. Moreover, OHCs are the most vulnerable cellular target in deafness caused by aging, acoustic injury and ototoxic drugs (Henderson et al., 2008; Ohlemiller and Frisina, 2008; Rybak et al., 2008). Therefore,

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studies of OHC physiology and pathology in vivo can be greatly benefited if inactivation of genes is inducible at specific times only in postnatal OHCs.

The Cre/loxP system has been widely applied to generate tissue-specific knockout mice. Many Cre mouse lines have been established for inner ear hair cells (Chow *et al.*, 2006; Gao *et al.*, 2004; Tian *et al.*, 2006; Yang *et al.*, 2010a; Yang *et al.*, 2010b) (NIH Neuroscience Blueprint Cre Driver Network-Database http://www.credrivermice.org/database). These mouse lines expressing Cre at different developmental ages (embryonic to neonatal) are powerful tools to study gene function in inner ear hair cells. Although these Cre lines are specific to hair cells in the cochlea, they can also express Cre in other tissues. Furthermore, the recently reported hair cell Cre lines Atoh1-Cre and Gfi1-Cre cannot be induced in a temporally controlled manner (Yang *et al.*, 2010a; Yang *et al.*, 2010b). Atoh1-CreER™ (Cre recombinase fused with estrogen receptor or ER) transgenic mice can be induced postnatally to control the Cre activity in cochlear hair cells. However, Atoh1-CreER™ is downregulated after P7 in cochlear hair cells (Chow *et al.*, 2006). Moreover, no OHC-specific Cre or CreER lines have been reported.

In the present study, we generated prestin-CreER^{T2} knockin mice that specifically express inducible Cre recombinase in OHCs at postnatal and adult ages. Prestin is first turned on at postnatal day 0–2 (P0–2) and maintained at high levels in adult OHCs. We generated 2 prestin knockin mouse lines: 1) prestin-CreER^{T2}, in which an internal ribosome entry site (IRES)-CreER^{T2}–FRT-Neo-FRT cassette is inserted into the prestin locus after the stop codon; and 2) prestin-CreER^{T2}-NN, in which the FRT-Neo-FRT cassette is subsequently removed from prestin-CreER^{T2}. The spatiotemporal inducible activity of Cre recombinase in both lines was examined by crossing prestin-CreER^{T2} mice with the CAG-eGFP or Ai6 reporter mice (Madisen *et al.*, 2010), and the hearing thresholds of mice were also tested. We found inducible prestin-CreER^{T2} activity specifically in postnatal developing and mature OHCs. These mouse lines are therefore valuable tools for examining gene functions in OHCs.

RESULTS AND DISCUSSION

Generation of the prestin-CreER^{T2} knockin mouse line

The prestin-CreER^{T2} knockin mouse line was created by using ES cell gene targeting and FLP-FRT technology (Fig. 1a). CreER^{T2} is a modified version of CreER™ (Feil *et al.*, 1997; Indra *et al.*, 1999); FRT is the recognition site for Flp recombinase (Dymecki, 1996); and Neo is the selection marker for the ES cell screen. A cassette with an internal ribosome entry site (IRES)-CreER^{T2} and the Neo marker flanked by 2 FRT sites was inserted into the prestin locus after the stop codon (Fig. 1a). After homologous recombination, correctly targeted ES cells with IRES-CreER^{T2} and the Neo marker were confirmed by Southern blotting. Forty-five (~14%) of 325 ES cell clones showed correct homologous recombination. Five colonies were chosen from these 45 colonies for karyotyping and 3 colonies with normal diploid were chosen for injection into blastocysts. High-level chimeras were chosen to obtain germline transmission. By using the 3′ external probe, it was confirmed that the germline-transmitted prestin-CreER^{T2} knockin mice were correctly targeted by Southern blotting analysis of expected DNA fragments 23kb (wild type) and 10kb (targeted) from *Spe*I-digested tail genomic DNA (Fig. 1b). Also, PCR genotyping showed a wild-type allele (300 bp) and the prestin-CreER^{T2} allele (228-bp) (Fig. 1c). Both the prestin-CreER^{T2} +/− (heterozygous) and prestin-CreER^{T2} +/+ (homozygous) mice were viable and had no detectable developmental defects.

OHC-specific, inducible Cre activity of prestin-CreER^{T2} knockin mice

To investigate the Cre recombinase inducible activity and tissue specificity of prestin-CreER^{T2} mice, prestin-CreER^{T2} +/− mice were bred with mice from reporter lines CAG-eGFP (Okabe *et al.*, 1997) and Ai6 (Madisen *et al.*, 2010). The eGFP or ZsGreen reporter gene is expressed only after Cre-mediated excision of a loxP-flanked stop cassette and the level of eGFP or ZsGreen expression is dictated by the CAG or ROSA26/CAG promoter, respectively. To determine CreER^{T2} activities are inducible, prestin-CreER^{T2};Ai6 mice were first intraperitoneally (IP) injected with tamoxifen at a dose of 3mg/40g body weight from P2 to P4. P7 cochleae from tamoxifen-injected mice displayed a strong green fluorescence in almost all OHCs (Fig. 2d–f), but those from mice not injected with tamoxifen did not (Fig. 2a–c).

Although in the developing cochlea, prestin can be detected as early as P0 (Belyantseva *et al.*, 2000), we found no detectable reporter expression in the prestin-CreER^{T2} +/− cochleae at P6 when tamoxifen (at 3mg/40g body weight) was injected at P0. However, when tamoxifen (at 3mg/40g body weight) was injected once daily at P0–P2, prestin-CreERT2 +/− cochleae showed an increasing gradient in reporter expression from apex (16.9±5.1%), middle (35.7±3.2%), to base (58.4±6.7%) when analyzed at P6 (Fig. 3a–c‴). This gradual Cre expression pattern is consistent with endogenous prestin expression in different turns at P0–P2 (Legendre *et al.*, 2008). After P2, almost all OHCs expressed the reporter with 2–5 continuous once-daily injections of tamoxifen (3mg/40g body weight) at different ages (Table 1). Only P10 apical-turn images from prestin-CreER^{T2} +/−;Ai6 mice induced tamoxifen at P5–7 are shown here because all image were similar at all turns and ages after P2 (Fig. 3d-d‴).

To reduce tamoxifen injection frequencies while achieving maximal (100%) Cre activity in OHCs, we increased tamoxifen doses to 9mg/40g body weight for adult mice. Cre activity was detected in all OHCs after a once-daily injection for 2 consecutive days.

Although prestin is highly specific for OHCs in the cochlea, it is expressed at low levels in the brain and testis (Zheng *et al.*, 2003) and vestibular hair cells (Adler *et al.*, 2003). Therefore, we checked for the presence of Cre activity in vestibular organs, testis, and brain in Prestin-CreER^{T2}; CAG-eGFP mice. There is no tamoxifen-induced Cre activity in these tissues (data not shown). Either prestin level is too low, or the IRES cassette before the CreER^{T2} may reduce Cre expression in these tissues.

Normal hearing in prestin-CreER^{T2} mice

Prestin KO and prestin nonfunctional mutant mice demonstrate that prestin is vital for OHC function and hearing (Dallos *et al.*, 2008; Liberman *et al.*, 2002). To determine whether the prestin-CreER^{T2} line maintained normal hearing, auditory brainstem responses (ABRs) were tested at adult ages. Compared with wild-type mice, prestin-CreER^{T2} +/− and prestin-CreER^{T2} +/+ mice showed no hearing defects at 4 kHz to 32 kHz at 4–6 weeks (Fig. 4).

Inducible Cre activity and hearing threshold of prestin-CreER^{T2}-NN mice

To determine whether the FRT-Neo-FRT cassette insertion had an effect on prestin-CreER^{T2} expression (Fig. 1a), we removed the FRT-Neo-FRT cassette by crossing prestin-CreER^{T2} mice with a ACTG-Flpe deletor (Rodriguez *et al.*, 2000) and determined inducible Cre activity in cochleae of the resulting prestin-CreER^{T2}-NN +/− mice that had deleted Neo and had no Flpe. These mice showed inducible Cre activity specific for all OHCs when the same tamoxifen injection protocols were used to prestin-CreER^{T2} mice (Table 1). All OHCs from Ai6; prestin-CreER^{T2}–NN +/− mice had Cre activity with tamoxifen (9mg/40g body weight) induction at P21–P22 when analyzed at P28 (Fig. 5a–f). ABR measurements of

prestin-CreER^{T2}-NN +/− mice showed that they had normal hearing at all frequencies. Although 7 of 11 prestin-CreER^{T2}-NN +/+ mice had threshold elevation 25–30dB at 16 and 22 kHz, the remaining 4 we tested had normal hearing (Fig. 5g). One possible reason for such variable high frequency threshold elevation in prestin-CreER^{T2}–NN +/+ mice is the substantial contribution of C57BL6 strain background from the ACTG-Flpe deletor in the mixed background of prestin-CreER^{T2}–NN +/+ mice (Davis *et al.*, 2007; Rodriguez *et al.*, 2000; Spongr *et al.*, 1997; Zheng *et al.*, 1999). Regardless, most applications use either prestin-CreER^{T2}–or prestin-CreER^{T2}–NN +/− that all display normal hearing.

In conclusion, our results demonstrate that both prestin-CreER^{T2} and prestin-CreER^{T2}-NN knockin mouse lines can be useful tools in manipulating gene function in postnatal and mature OHCs. Because of the strong inducible Cre activity in almost all OHCs at postnatal and adult ages, both the prestin-CreER^{T2} and prestin-CreER^{T2}-NN +/− background display normal hearing, thus suitable for OHC-specific gene manipulations in most applications, and these mice will exhibit no additional hearing defects from the prestin locus. Moreover, when induced at P0–2, the inducible Cre activity of these mice displayed a gradient of reporter expression from the apex to base of the cochlea (Fig. 3a–c‴); such a mosaic gradient pattern of Cre activity could be useful for specific applications of OHC gene function analysis in vivo.

Materials and methods

Generation of prestin-CreER^{T2} knockin mice

A prestin bacterial artificial chromosome (BAC) was used for modification to generate targeting vector by the highly efficient recombineering-based method (Liu *et al.*, 2003). The prestin-CreER^{T2} targeting vector was constructed by inserting IRES-CreR^{T2}-FRT-Neo-FRT cassette after the stop coden in exon 20. The targeting vector, constructed in PL253 (Fig. 1a), was electroporated into 129/SvEvTac (129S6) ES cells (TL-1). For prestin-CreER^{T2} mice PCR genotyping, a PCR assay was developed with 3 primers: 5′-CACAAGTTGTGAATGACCTC -3′, 5′-GTTAAAGAGCGTAATCTGGAACA- 3′, and 5′-TAACTGCTAGCATTTCCCTT- 3′. To remove the neo cassette, prestin-CreER^{T2} +/− mice were bred with ACTFlpe mice. Prestin-CreER^{T2}–NN indicated the prestin-CreERT2 no neo cassette. Prestin-CreER^{T2}–NN +/− without Flpe mice were obtained by PCR genotyping and inducible Cre activity was further analyzed in these mice. The neo PCR genotyping protocol has been described in Parker et al (Parker *et al.*, 2006).

Ai6, CAG-eGFP conditional reporter, and ACTFlpe mice were obtained from The Jackson Laboratory (Stock# 007906, Stock # 003291, and Stock# 003800, respectively) and PCR genotyping of the reporter mice were performed according to protocols provided by The Jackson Laboratory.

Tamoxifen injection

Tamoxifen (Sigma, St. Louis, MO) was dissolved in corn oil (Sigma) at a concentration of 5 mg/mL or 15 mg/mL at 37°C (approximately 1 h). Tamoxifen was filter-sterilized and stored at 4°C in the dark for up to 7 days. Pups were intraperitoneally injected by a 30G1/2(Thinner 3/10ml) ultrafine needle insulin syringe (Becton Dickinson) and adult mice by a 26G3/8 needle insulin syringe. All different combinations of mice were injected for 2–5 consecutive days with tamoxifen at a dose of 3 mg/40 g body weight or 9 mg/40 g mouse weight (Table 1).

Histologic Analysis and Immunocytochemistry

Mice were anesthetized with avertin and fixed by intracardial perfusion with 2%–4% paraformaldehyde (PFA). Whole-mount immunolabeling was performed as previously described (Liu *et al.*, 2010). Briefly, whole-mount cochlear coils were dissected and labeled with anti-prestin (goat, 1:200, Santa Cruz Biotechnology, sc-22692); anti-myo7a (1:200, Proteus Bioscience, 25-6790). Alexa-conjugated secondary antibodies (Invitrogen) were used at a concentration of 1:1000 (donkey anti-rabbit Alexa Fluor 647, donkey anti-goat Alexa Fluor 568). Samples were mounted in mounting medium with DAPI (Vectashield; Vector Laboratories, Burlingame, CA).

Auditory brainstem response (ABR)

Auditory brainstem response (ABR) measurements were performed as previously described (Wu *et al.*, 2004).

Statistical analyses

GraphPad Prism (GraphPad Software, San Diego, CA) was used for statistical analysis. Data were analyzed using two-way ANOVA, followed by a Student's t test with a Bonferroni correction.

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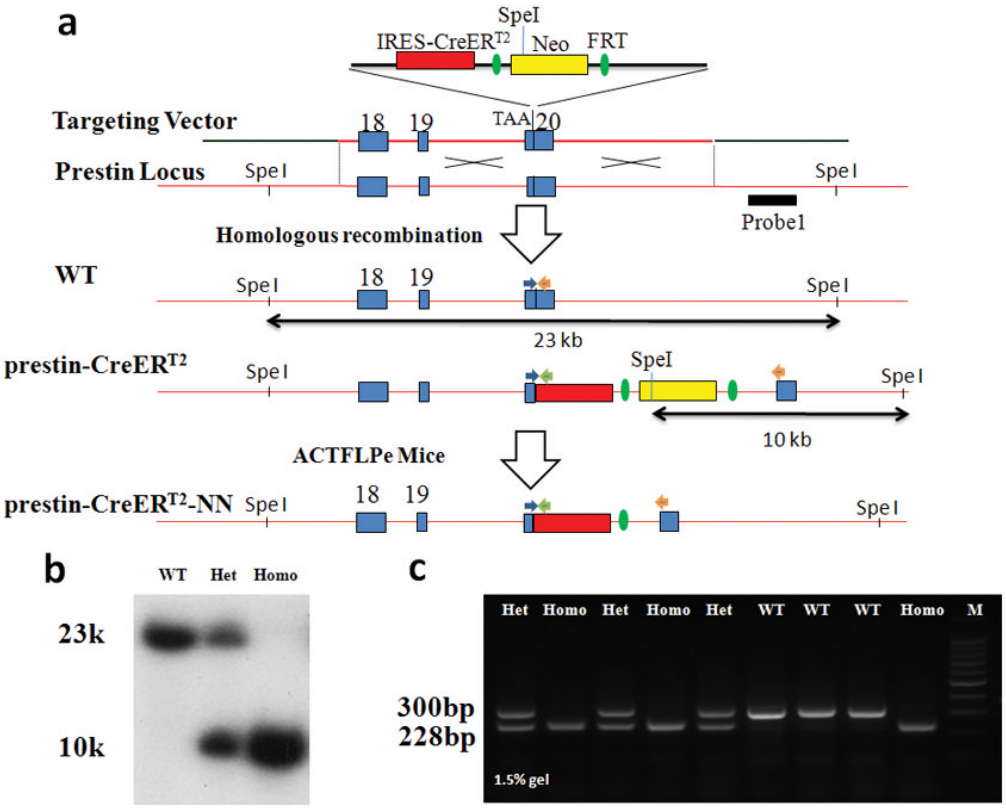

FIG. 1. Generation of prestin-CreER^{T2} knock-in mice

(a) Schematic diagram of generation of prestin-CreER^{T2} +/− and prestin-CreER^{T2}–NN (No Neo cassette) knock-in mice. Solid blue rectangles represent exon18–20 of the prestin gene. The external 3′-Southern probe is shown as the black bar. Arrows indicate the approximate positions of the PCR genotyping primers. Abbreviations: IRES, internal ribosome entry site; CreER^{T2}, Cre recombinase fused to a mutant estrogen ligand-binding domain; Neo, PGK-neo cassette; FRT, FLP recombinase target.

(b) Southern blot genotyping analysis of prestin-CreER^{T2} +/− and prestin-CreER^{T2} +/+ mice. Genomic tail DNA was digested with *Spe*I and a 3′-external probe indicated in (a) was used to identify the 23-kb wild-type and the 10-kb targeted DNA fragments.

(c) PCR genotyping shows 300-bp product (wild type) and 228-bp product (prestin-CreER^{T2} +/− mice).

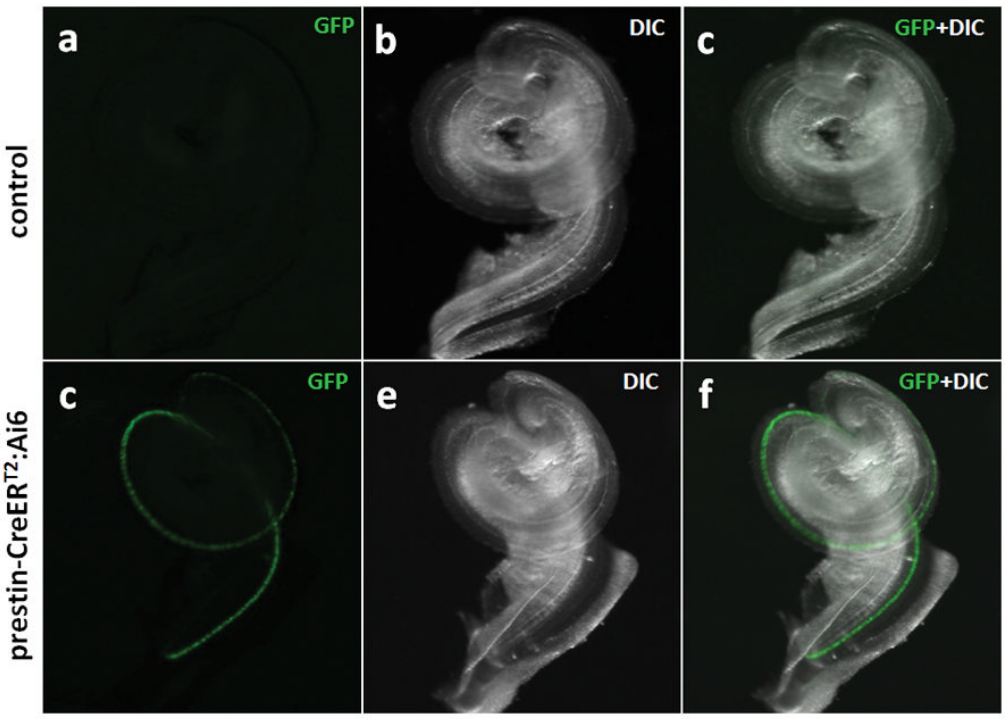

FIG. 2.

Inducible Cre recombination in prestin-CreER^{T2} +/− mice. Images of a whole cochlea (P7) from a prestin-CreER^{T2} +/−; Ai6 mouse without (a–c) and with (d–f) tamoxifen injection at P2–4.

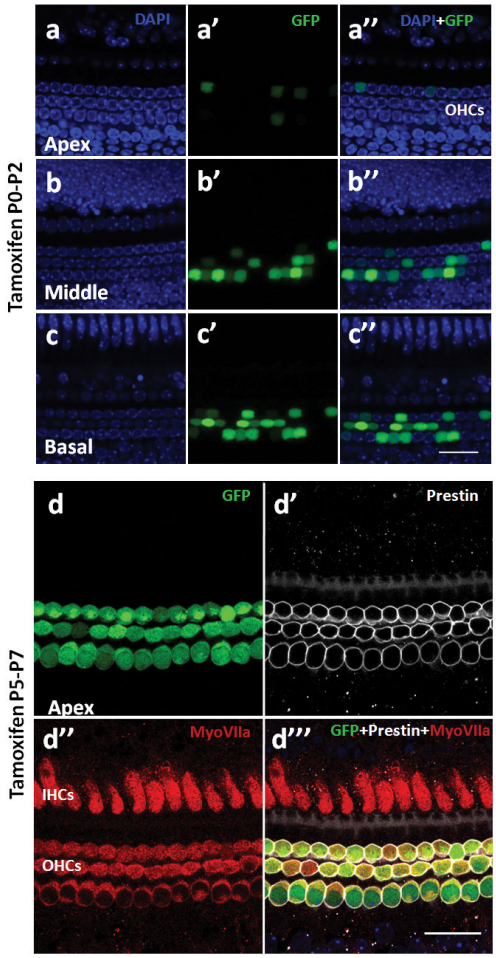

FIG. 3.

Cre recombination in all OHCs at various postnatal ages. (a-c″) prestin-CreER^{T2} +/− shows a gradual decrease pattern in reporter expression. prestin-CreER^{T2} +/−; CAG-eGFP mice were injected with tamoxifen (3mg/40g body weight) at P0–2. (d-d‴) prestin-CreER^{T2} +/−; Ai6 mice were injected with tamoxifen at P5–7. Images of GFP (d), anti-prestin (d′), anti-myo VIIa (d″), and merger (d‴). IHCs: Inner hair cells, OHCs: outer hair cells. Scale bar: 20 μm.

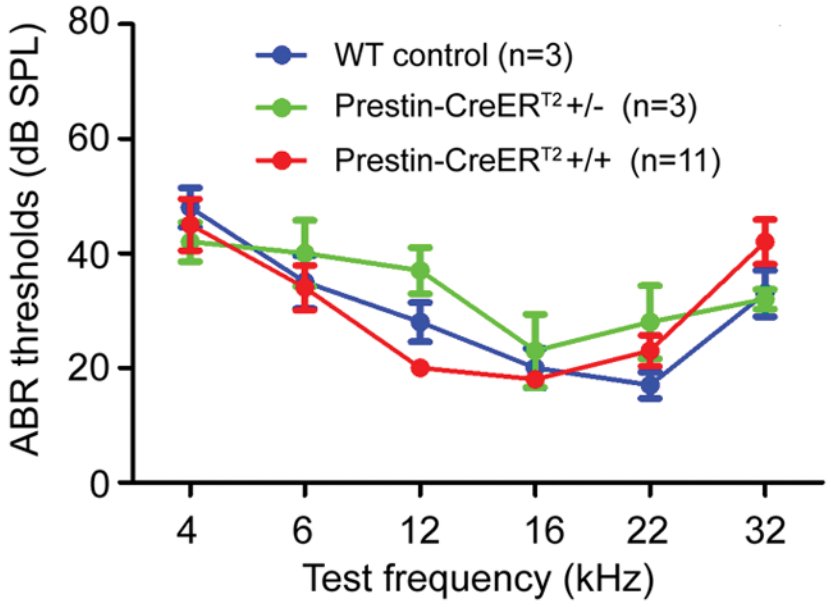

FIG. 4.

Auditory brainstem response (ABR) thresholds of prestin-CreER^{T2} +/− and prestin-CreER^{T2} +/+ and wild-type mice at 4–6 weeks. *n*, number of mice for ABR testing for each genotype. Bars: standard error of mean.

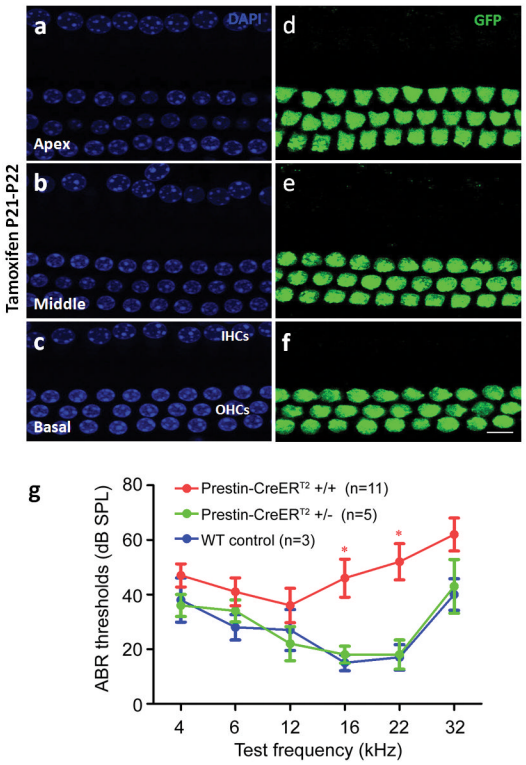

FIG. 5.

Cre activity and hearing ability of prestin-CreER^{T2}-NN +/− mice. Images of P28 prestin-CreER^{T2}-NN +/−; Ai6 mouse inducted tamoxifen (9mg/40g) at P21–22, DAPI (a, b, c, at the nuclear level) and GFP (d, e, f at the cell body level), in each cochlear turn. Scale bar: 20 μm. (g) ABR thresholds of prestin-CreER^{T2}-NN +/−, prestin-CreER^{T2}-NN +/+ and wild-type mice at 4–6 weeks. *N*, number of mice for ABR testing for each genotype. Bars: standard error of mean. *: $P < 0.05$.

Table 1

Injection schedule for inducible Cre activity in all OHCs

Tamoxifen (once per day)	Injection day	Analysis day
3mg/40g body weight	P2–P4	P7–8
3mg/40g body weight	P5–P7	P10–12
3mg/40g body weight	P9–P10	P14–16
3mg/40g body weight	P14–P16	P20–P21
3mg/40g body weight	P21–P25	P28–P30
9mg/40g body weight	P21–P22	P25–28