PIEZ02 mediates ultrasonic hearing via cochlear outer hair cells in mice

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Ultrasonic hearing and vocalization are the physiological mechanisms controlling echolocation in hunting and navigation by microbats and bottleneck dolphins and for social communication by mice and rats. The molecular and cellular basis for ultrasonic hearing is as yet unknown. Here, we show that knockout of the mechanosensitive ion channel PIEZ02 in cochlea disrupts ultrasonic hearing but not low-frequency hearing in mice, as shown by audiometry and acoustically associative freezing behavior. Deletion of PIEZ02 in outer hair cells (OHCs) specifically abolishes associative learning in mice during hearing exposure at ultrasonic frequencies. Ex vivo cochlear Ca2+ imaging has revealed that ultrasonic transduction requires both PIEZ02 and the hair-cell mechanotransduction channel. The present study demonstrates that OHCs serve as effector cells, combining with PIEZ02 as an essential molecule for ultrasonic hearing in mice.

Results

The Mechanosensitive Channel PIEZ02 Is Required for Ultrasonic Hearing. To evaluate ultrasonic hearing physiologically, we improved the basic ABR recordings by attaching the electrode directly to the skull bone positioned posterior to Bregma sutures and finally disparates at approximately postnatal 7 d (P7) (19, 20), which is opposite the maturation of hair-cell mechanotransduction current (19, 21). In adult mice, knockout (KO) of Piez2 in the inner ear only slightly affects hearing from 8 to 20 kHz as demonstrated by auditory brainstem response (ABR) recordings (18). To date, the physiological role of PIEZ02 in hearing is still not established (20).

In this study, we explored the role of PIEZ02 in ultrasonic hearing from a variety of KO and conditional KO (cKO) mouse lines, using ultrasonically combined approaches, including ABR recordings, behavior tests, and ex vivo cochlear imaging assays. We found that the expression of PIEZ02 in the OHCs is essential for ultrasonic hearing in mice.

Some animals use ultrasonic hearing and vocalization to communicate and navigate in daily life (1). For example, mice vocalize at frequencies >25 kHz with intensities from 60 to 100 dB SPL (sound pressure level) during certain social behaviors, including mother–pup interactions, male–male encounters, and male–female courtship (2–4). Thus, ultrasound-based auditory communication is critical for the survival and reproduction of mice. Animal models using mice, bats, cats, and guinea pigs have provided neurophysiological insights into ultrasonic hearing (5–9). However, the precise molecular identity and cell type defining ultrasonic transduction are not known. Current scientific thought postulates that ultrasonic hearing shares well-recognized general molecular and cellular mechanisms that produce auditory transduction (10–13). However, the paucity of scientific evidence in this area of hearing research relegates these theories to unproven speculation.

Recently, it has been reported that the mechanosensitive ion channel PIEZ02 plays a critical role in the somatosensory system, including gentle touch, itch, tactile pain, proprioception, breath, and blood pressure (14, 15). Structural and functional analyses of PIEZ02 have shown that it can respond to different forms of mechanical stimuli, such as indentation and stretching (16, 17). Interestingly, PIEZ02 was found expressed at the apical surface also known as the cuticular plate of cochlear hair cells, mainly in outer hair cells (OHCs) (18). It mediates a stretch-activated current known as the reverse-polarity current in neonatal mice and is controlled by intracellular Ca2+ concentration (18, 19). However, this electrical current gradually reduces with age and finally disperses at approximately postnatal 7 d (P7) (19, 20), which is opposite the maturation of hair-cell mechanotransduction current (19, 21). In adult mice, knockout (KO) of Piez2 in the inner ear only slightly affects hearing from 8 to 20 kHz as demonstrated by auditory brainstem response (ABR) recordings (18). To date, the physiological role of PIEZ02 in hearing is still not established (20).

In this study, we explored the role of PIEZ02 in ultrasonic hearing from a variety of KO and conditional KO (cKO) mouse lines, using ultrasonically combined approaches, including ABR recordings, behavior tests, and ex vivo cochlear imaging assays. We found that the expression of PIEZ02 in the OHCs is essential for ultrasonic hearing in mice.

Significance

Some animals have evolved an adaptive ability for vocalizing and hearing ultrasonic frequencies that are inaudible to humans (>20 kHz). For decades, it has been postulated that animals hear ultrasonic frequencies with cochlear hair cells using an identical set of mechanotransduction molecules in the hair bundles for hearing audible frequencies. Here, we show that mice lacking mechanosensitive PIEZ02 ion channels have difficulty hearing at ultrasonic frequencies but remain remarkably sensitive to audible frequencies. Thus, animals may use a partially different mechanism for sensing ultrasonic sound emissions.


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stimuli at frequencies >12 kHz in C57BL/6 (B6) mice (Fig. 1 B and C). Although ultrasonic responses were not as strong as those induced by low frequencies, the mABR waveforms induced by ultrasonic frequencies were readily distinguishable for determining

Fig. 1. The mechanosensitive channel PIEZO2 is required for ultrasonic hearing. (A) Schematic of recording configuration of mABR. For mABR, the recording wire was directly implanted on the mouse skull (cyan) instead of placing a needle electrode under scalp (dark yellow) (reference SI Appendix, Fig. S1). (B) Representative example of mABR signals in a C57BL/6J (B6) mouse. (C) Comparison of regular ABR in B6 mice with attached electrode under scalp (scalp B6, dark yellow); in B6 mice, the mABR achieved improved sensitivity to frequencies >12 kHz (modified B6, cyan). Threshold of scalp B6 and modified B6 were compared by Mann–Whitney U test at each frequency, 4 kHz, *P = 0.0146; 8 kHz, P = 0.4432 (no significance, ns); 12 kHz, **P = 0.0028; 16 kHz, ****P < 0.0001; 32 kHz, ****P < 0.0001. (D) Representative example of mABR signals in Piezo2-cKO mouse. (E) Enlarged traces with 63 and 80 kHz sound stimuli framed in B and D. (F) Pure-tone mABR thresholds in control Pax2Cre mice and Piezo2-cKO mice. The control- and Piezo2-cKO mice showed distinct ABR thresholds at ultrasonic frequencies (gray-shaded area). Kruskal–Wallis test was used to compare the three groups at each frequency. Pax2Cre mice versus Pax2Cre; Piezo2f/f cKO mice, 4 kHz, P = 0.9999; 8 kHz, P = 0.9999; 12 kHz, P = 0.9999; 16 kHz, *P = 0.0214; 32 kHz, ****P < 0.0001; 40 kHz, ****P < 0.0001; 54 kHz, ****P < 0.0001; 63 kHz, ****P < 0.0001; 80 kHz, ****P < 0.0001. Pax2Cre mice versus Atoh1Cre; Piezo2f/f cKO mice, 4 kHz, P = 0.122; 8 kHz, P = 0.956; 12 kHz, P = 0.8272; 16 kHz, *P = 0.025; 32 kHz, ****P < 0.0001; 40 kHz, *P = 0.0167; 54 kHz, ****P = 0.0017; 63 kHz, ****P = 0.0026; 80 kHz, ****P = 0.0002. All mice were recorded at ∼1 mo old. For C and F, data are presented as mean ± SD, and N numbers are shown in panels.
thresholds (Fig. 1B). The generally decreased mABR amplitude at 63 and 80 kHz implies less efficient ultrasonic transduction at the cochlear level. This is because the ABR waveforms reflect signals from the auditory nerves that innervate the cochlear hair cells and their ascending auditory pathways (22). The 54-kHz 90 dB SPL mABR signal showed an abruptly large amplitude (Fig. 1B). This is consistent with previous measurements of mouse hearing, using audiometry (23) and auditory nerve recordings (24), which showed two sensitivity peaks at 15 and 55 kHz, respectively. This phenomenon was not due to distortions delivered by the speakers at high intensities, since the measured ultrasonic pure-tone output was quite condensed even at 90 dB SPL (SI Appendix, Fig. S1D).

Since PIEZO2 pan-KO mice exhibit embryonic lethality (25), we crossed certain Cre mice with Piezo2f/f mice (26) to generate Piezo2-cKO mice. These mice were mainly of B6- and some mixed genetic background (SI Appendix, Table S1). It has been shown that mice with different backgrounds have different ultrasonic hearing sensitivities. For example, the CBA/J (CBA) mice have better ultrasonic hearing sensitivity compared to B6 mice according to the auditory nerve recordings (8 to 16 wk animals) (24) and ABR recordings (16 to 18 wk animals) (27). In addition, B6 mice exhibit progressive hearing loss late in life (>7 mo) (28, 29). To avoid the possible statistical confound of mixed genetic background in the evaluation of ultrasonic hearing, we compared the mABR sensitivity between the B6 mice and the CBA mice at ~1 mo old. This is the age at which they possess matured auditory function, occurring well before age-related hearing loss begins. The mABR recordings showed that ultrasonic hearing in B6 mice is as sensitive as that of CBA mice at the age of 1 mo old (SI Appendix, Fig. S1E). In addition, male and female mice showed similar mABR thresholds (SI Appendix, Fig. S1F).

Next, we evaluated hearing and auditory transduction in hybrid Piezo2-cKO mice. Unless otherwise stated, their littermates were used as controls (~1 mo). To check whether PIEZO2 participates in ultrasonic hearing, we compared mABR of inner-ear targeted Pax2Cre;Piezo2f/f mice (18, 30) and cochlea targeted Piezo2-cKO mice by crossing the Piezo2f/f mice with Atoh1Cre mice (31). The control mouse showed notable mABR signals at 32 to 80 kHz (Fig. 1B), while the Pax2Cre;Piezo2f/f cKO mouse showed decreased response at frequencies >32 kHz (Fig. 1D). This difference is clearly seen when comparing their ABR responses taken together (Fig. 1E). The summarized mABR recordings revealed that two types of Piezo2-cKO mice both had significantly reduced specific sensitivity to ultrasonic hearing (16 to 80 kHz) but not to low-frequency hearing (4 to 12 kHz) (Fig. 1F). Thus, these data provide convincing evidence that PIEZO2 is required for ultrasonic hearing.

In addition, we examined whether lack of ultrasonic hearing is due to loss of the “high-frequency” hair cells at the very basal coil of cochlea in the Piezo2-cKO mice. No obvious loss of hair cells was found in the cochleae of the Pax2Cre;Piezo2f/f cKO mice, which revealed preserved normal morphology of the hair cells (SI Appendix, Fig. S2). We further examined the organization of the inner ear of the Pax2Cre;Piezo2f/f cKO mouse by a tissue-clearing approach (32) (Materials and Methods). The whole structure of the inner ear was intact with hair cells remaining in normal allocation and abundance (Movies S1 and S2).

**Ultrasonically Associative Freezing Behavior Is Disrupted in Piezo2-cKO Mice.** We investigated whether PIEZO2-mediated ultrasonic hearing sensitivity is required for learned behavior in mice. The hearing response of the Piezo2-cKO mouse was examined by a fear conditioning test (Fig. 2A), which associates an acoustic cue to the freezing behavior in mice, after paired training of acoustic cues with electrical shocks. A 90-dB SPL ultrasonic 63-kHz stimulatory cue was chosen because the 63-kHz mABR showed a stable threshold difference between control and Piezo2-cKO mice (Fig. 1F). Note that 63 kHz is in the range of ultrasonic frequencies of mouse social communications (1).

To exclude the possibility that disrupted ultrasonic-associative fear conditioning was due to a learning defect in Piezo2-cKO mice, we repeated the same experiments in control animals under identical conditions, testing freezing behavior with a 16-kHz 90-dB SPL cue. We found that harmonics appear in the 16-kHz 63-kHz comparison of freezing behavior to the 90-dB 16-kHz cue for Piezo2-cKO mice. (A) Paradigm of sound-cue-associated freezing behavior. Pure-tone sound at 16 or 63 kHz played by a TDT ES1 (free field) electrostatic speaker was used as the conditional stimulation, and footshock was used as the unconditional stimulation. (B) Representative examples of locomotion of control mice and Piezo2-cKO mice before (gray, 30 s), during (red, 10 s), and after (blue, 30 s) the pure-tone sound cue at 90 dB SPL. The mice had been trained to pair either 16-kHz cue or 63-kHz cue with the footshock-induced freezing. Dots indicate the location of the mouse every 0.5 s. Pax2Cre; Piezo2f/f; Atoh1Cre; Piezo2f/f mice were used as controls (C and D). Freezing time in percentage is shown with 16-kHz cue (C) or 63-kHz cue (D). In D, experimental data from two Pax2Cre; Piezo2f/f mice were omitted because during testing, no locomotion was detected in these animals. Kruskal–Wallis test, P = 0.225 in C; one-way ANOVA, ****p < 0.0001 in D; error bars, SD. For C and D, N numbers are shown in panels. All the mice were ~1 mo old.
and *Atoh1* Cre;Piezo2 f/f cKO mice showed that all genotypes retained their low-frequency–associative freezing behavior (Fig. 2B). This is illustrated by the freezing time percentage (Fig. 2C), indicating that these mice possess low-frequency hearing and acoustically associative learning. In contrast, when exposed to the 63-kHz cue, the Pax2 Cre;Piezo2 f/f and *Atoh1* Cre;Piezo2 f/f cKO mice showed disrupted freezing behavior (Fig. 2B and D). We observed that ultrasonically conditioned learned behavior was preserved in the control mice (Fig. 2D). These data demonstrate that PIEZO2 is required for mice to behaviorally respond to ultrasound.

**Expression of PIEZO2 in Cochlear Hair Cells.** Next, we examined the expression of PIEZO2 in the cochlea. First, the Pax2-GFP-IREs-Cre mice that carry a Cre cassette with the endogenous Piezo2 (26) were crossed with the H2B-mCherry mice that have Cre-inducible mCherry expression (33). As previously reported (18), the mCherry expression was observed in most OHCs and some inner hair cells (IHCs) in 1-mo-old mice (SI Appendix, Fig. S4 A–D). This indicates that the Piezo2 promoter is transcriptionally active in cochlear hair cells. Due to lack of suitable PIEZO2 antibody, we used GFP antibody to locate PIEZO2 expression in the Piezo2-GFP-IREs-Cre mice that is comprised of a GFP gene fused with Piezo2. As reported earlier (18), the PIEZO2 immunostaining signal was mainly detected at the apical surface of cochlear OHCs by the GFP antibody at P5 (Fig. 3A). However, this signal was faint and difficult to detect at more mature ages (∼3 to 4 wk old). Thus, we applied an RNAscope protocol (34) to check the expression of Piezo2 transcript in control and Pax2 Cre;Piezo2 f/f mice at P21. Within the cross-section of the organ of Corti, the Piezo2 transcript was observed in the control Piezo2 f/f hair cells though at relatively low levels, while the transcript was more significantly reduced in Pax2 Cre;Piezo2 f/f hair cells (Fig. 3 B and C). Control staining in either the inner ears or the dorsal root ganglia was performed to validate the effectiveness of the optimized RNAscope procedure (SI Appendix, Fig. S4 E and F). These data indicate that PIEZO2 is in fact expressed in the cochlear hair cells of adult mice.

**Mice Lack Ultrasonic Hearing When Piezo2 Was Deleted in OHCs.** We investigated which type of hair cell supports PIEZO2’s role for ultrasonic hearing by examining the ultrasound-associative freezing behavior in mice when deleting Piezo2 in OHCs or IHCs. Prestin CreER mice (35) were introduced to generate OHC-specific Piezo2-cKO mice because Prestin is only expressed in OHCs (36). vGlut3 CreER;Piezo2 f/f mice were used to check PIEZO2’s role in IHCs (37). To validate the specificity of hair-cell expression, Prestin CreER;H2B-mCherry mice and the vGlut3 CreER;H2B-mCherry mice were injected with tamoxifen at P8 to P10, a time with peaked expression of Prestin and vGlut3. Later, these mice were examined for mCherry expression at 1 to 2 mo old (SI Appendix, Fig. S5A). In Prestin CreER;H2B-mCherry mice, only the OHCs showed high efficiency mCherry expression (SI Appendix, Fig. S5B). By comparison, mCherry was widely expressed in IHCs of the vGlut3 CreER;H2B-mCherry mice (SI Appendix, Fig. S5C).

Using the same injection procedure (Fig. 4A), induced Prestin CreER;Piezo2 f/f cKO mice at 1 mo old showed freezing behavior when exposed to low-frequency stimulation but not to the ultrasonic cue (Fig. 4 B and C). This indicates a necessary

![Fig. 3. Expression of Piezo2 in cochlear hair cells. (A) Schematic showing hair-bundle layer (yellow) and cuticular plate (blue) of an outer hair cell. Piezo2 signal (green) was detectable at cuticular plate (Lower in blue frame, white arrows) but not in hair bundles (Upper in yellow frame), as stained by GFP antibody (green) in P5 Piezo2-GFP mice. Hair bundle was stained by Phalloidin (red). Scale bar, 20 μm.) (B) Cross-sections of organ of Corti of a Piezo2 f/f mouse and a Pax2 Cre;Piezo2 f/f mouse at P21 showing fluorescent signals of RNAscope probe targeting Piezo2 (red dots, indicated by arrows). Some dots were out of focus and not well visualized. Hair cells and nuclei were stained with MYO7A antibody (green) and DAPI (blue), respectively. (Scale bars, 20 μm.) (C) Quantification of Piezo2 dots in cochlear hair cells from data similar to B. Cochlear sections were collected from P21 mice for each genotype. Note that multiple hair cells were superimposed. Numbers of transcript dots were counted per section, and numbers of hair cells were counted based on the MYO7A and DAPI signals. Unpaired t test with Welch’s correction, OHCs, **P = 0.0026; IHCs, P = 0.1233. Error bars, SD. N numbers of sections are shown in panels.](https://doi.org/10.1073/pnas.2101207118)
requirement of OHCs for ultrasonic hearing. On the contrary, the \textit{vGlut3\textsuperscript{CreER}, Piezo2\textsuperscript{f/f}} mice did not show any deficit of ultrasound or low-frequency sound-associative freezing (Fig. 4 D and E), excluding a possible role of IHCs in PIEZO2-mediated function in ultrasound detection. These data confirm that expression of PIEZO2 in cochlear OHCs is required for ultrasonic hearing.

Hair-Bundle Mechanotransduction Is Required for Ultrasonic Transduction.

The OHCs are capable of augmenting cochlear mechanics that enhance hearing sensitivity and frequency selectivity (38, 39). This is supported by electromotility (eM), a function that changes somatic cell shape (cellular length) with membrane potential variations in OHCs (38, 39). Thus, we sought to investigate the OHC eM in Piezo2-cKO mice by examining nonlinear capacitance (NLC) (40). In response to electrical stimuli, no obvious eM deficiency was found in Pax2\textsuperscript{CreER}, Piezo2\textsuperscript{f/f} OHCs (SI Appendix, Fig. S6), which is in line with previous measurements (18).

Next, we questioned whether hair-bundle mechanotransduction participates in ultrasonic transduction. It has been reported that TMC1 is the putative auditory transduction channel in hair cells, and CDH23 is one of the two tip-link components (41, 42). Thus, we used the TM1KO mice (43) and the CDH23-null \textit{vGlut3}\textsuperscript{CreER}, Piezo2\textsuperscript{f/f} mice (44) to investigate their ultrasonic transduction. As shown by MABR recordings at 1 mo old, the two mice mutants had complete loss of hearing, which ranged from low to ultrasonic frequencies (SI Appendix, Fig. S7A). This is likely the result of abnormal hair bundles (43, 44) and loss of the hair cells (SI Appendix, Fig. S7 B and C).

These results suggest that neither audiometry nor behavioral

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**Fig. 4.** Mice lack ultrasonic hearing when Piezo2 was deleted in OHCs. (A) Schedule of tamoxifen injection and behavior test. (B) Representative example of locomotion of \textit{Prestin\textsuperscript{CreER}, Piezo2\textsuperscript{f/f}} mice with or without tamoxifen injection. Different colors represent the locomotion before (gray), during (red), and after (blue) the 16-kHz or 63-kHz sound cue. Dots indicate the location of the mouse every 0.5 s. (C) Freezing time (in percentage) of the \textit{Prestin\textsuperscript{CreER}, Piezo2\textsuperscript{f/f}} mice trained and tested with 16-kHz cue (Left) or 63-kHz (Right) cue. The comparison was performed between the tamoxifen uninjected group and the tamoxifen injected group. Sixteen kHz, precue, unpaired \textit{t} test, \(P = 0.4468\); postcue, Mann-Whitney \textit{U} test, \(P = 0.513\); 63 kHz, precue, Mann-Whitney \textit{U} test, \(P = 0.4468\); postcue, unpaired \textit{t} test, **** < 0.0001. (D) Representative example of locomotion of \textit{vGlut3\textsuperscript{CreER}, Piezo2\textsuperscript{f/f}} mice with or without tamoxifen injection. Other conditions are the same with B. (E) Freezing time of the \textit{vGlut3\textsuperscript{CreER}, Piezo2\textsuperscript{f/f}} mice trained and tested with 16-kHz cue (Left) or 63-kHz (Right) cue. The comparison was performed between the tamoxifen uninjected group and the tamoxifen injected group. Sixteen kHz, precue, Mann-Whitney \textit{U} test, \(P = 0.3217\), postcue, unpaired \textit{t} test, \(P = 0.492\); 63 kHz, precue, Mann-Whitney \textit{U} test, \(P = 0.2052\), postcue, unpaired \textit{t} test, \(P = 0.203\). For C and E, data are presented as mean ± SD; and N numbers are shown in panels.
measures are proper probes for assessing the contribution of hair-bundle mecanotransduction. A more appropriate approach would be to use techniques targeting fine resolution of murine cellular structure and function.

To develop direct monitoring capability of ultrasasonic transduction in cochlear hair cells, we customized an ex vivo ultrasonic stimulation stage. It can deliver ultrasonic vibration of 80 kHz—a frequency within the range of the physiological hearing of mice (Materials and Methods and SI Appendix, Fig. S8 A–C). This ultrasonic stimulation mimics the mechanical vibration in the cochlea driven by incoming ultrasound. One obstacle to this experiment was obtaining an intact organ of Corti from mice after hearing onset because the cochlea has been embedded into the bony capsule of the inner ear. Instead, we introduced a hemicochlear preparation (45, 46) that preserves most of the elements of the cochlea and is also accessible to microscopic observation (Fig. 5A). Because patch-clamp recording of hair cells is routinely destroyed by direct ultrasonic stimulation, we used Ca2+ imaging for monitoring ultrasonically evoked activity (Fig. 5B).

The hemicochlear preparation was loaded with Fluoro-8 AM, a sensitive Ca2+ dye. The OHCs were the major cells with Ca2+ dye uptake (Fig. 5C). Ultrasonic stimulation elicited Ca2+ waves in the OHCs of wild-type (WT) hemicochlear despite apical or middle-positioning in the cochlear coil. This effect could be blocked when perfusing cells with 0.1 mM Ca2+ solution (SI Appendix, Fig. S8 D and E), which suggests that the OHCs are ultrasonically responsive. After ultrasonic stimulation, the Fluo-8–loaded OHCs showed an evoked Ca2+ wave when ATP (100 μM) was applied (SI Appendix, Fig. S8 D and E). This indicates that the OHCs were healthy after ultrasonic stimulation and that the Ca2+ response was not saturated.

We evaluated the role of PIEZO2 and TMC1 in ultrasonic transduction by hemicochlear Ca2+ imaging. Ultrasonic stimulation was capable of eliciting Ca2+ responses in OHCs of the control Piezo2+/+ mice. However, when Piezo2 was genetically removed from the OHC, Ca2+ response was significantly diminished (Fig. 5 D and E and Movies S3 and S4). Although OHCs were widely lost in Tmc1–/– mice from 3-wk-old litters (SI Appendix, Fig. S7B) (43), we could readily identify apical OHCs with clear soma shapes illuminated by Fluoro-8 for Ca2+ imaging. The ultrasonic Ca2+ response was significantly reduced in the Tmc1–/– OHCs or in WT OHCs blocked by the mecanotransduction channel-blocker dihydrotestosterone (DHS, 100 μM) (Fig. 5 F and G). This implies that hair-bundle mecanotransduction is a requirement in ultrasonic transduction.

To investigate the response evoked by low-frequency stimulation, we used fluid jet, a stimulation which directly deflects the hair bundle to induce the mecanotransduction channel-mediated Ca2+ response in Tmc1–/–KO or Pax2–/– Piezo2+/+ OHCs. Similar to ultrasonic frequencies, there is no fluid-jet-evoked Ca2+ response in Tmc1–/–KO OHCs. In contrast, the fluid-jet-induced Ca2+ response was maintained in Pax2–/– Piezo2+/+ OHCs and WT OHCs (Fig. 5 F and I). These ex vivo results showed that PIEZO2 is required for the OHC Ca2+ response stimulated by ultrasonic frequencies but not for those stimulated by low frequencies. We further tested whether PIEZO2 and/or TMC1 could establish the ultrasonic transduction in exogenous expression systems. Although the human embryonic kidney 293T (HEK293T) cells expressing PIEZO2 were mechanosensitive (SI Appendix, Fig. S9 A and B), we did not observe any response in the HEK293T cells expressing PIEZO2 or PIEZO2 plus TMC1 when using the same 80-kHz ultrasonic stimulation applied for hemicochlear imaging (SI Appendix, Fig. S9 C and D). These results show that PIEZO2 may coordinate with the hair-bundle mecanotransduction machinery to effectuate ultrasonic transduction.

Discussion

We have shown that multiple lines of evidence suggest that PIEZO2 is essential for mice hearing ultrasonic frequencies within the range necessary for social communication. Furthermore, ultrasonic transduction is mainly conducted by OHCs, which coordinates hair-bundle mecanotransduction. Small mammals, such as mice, emit strong ultrasonic vocalization for social communication while overriding ambient noise, or masking sound emissions, which escape potential detection by predators. This paper posits a putative molecular and cellular mechanism underlying ultrasonic hearing and transduction, which, heretofore, has not been established.

It has been shown that with low-level Piez1 expression, PIEZO1-mediated mechanically activated current can be detected in HEK293T–derived cell lines (47). PIEZO2’s structure and mechanosensitivity properties likely support its role for OHCs in ultrasonic transduction. Previous evidence has shown that in mice neonates, PIEZO2 is expressed in cochlear hair cells (18). Our findings suggest that PIEZO2 expression in OHCs continues to at least weeks 3 to 4 (Fig. 5 B and C). Piezo2 expression shown by RNAscope assays is low, which may not reflect the quantity of PIEZO2 in OHCs.

PIEZO2 is a mechanosensitive channel that homotrimersize to form a gigantic (~0.9 MDa) three-bladed propeller-like structure comprising 114 transmembrane (TM) domains (38 TM per pro- tomer), making it a unique membrane protein with the largest number of TMs (16). There are three strikingly unusual nonplanar TM blades that are curved into a nano-bowl shape of 28-nm diameter and 10-nm depth, which might deform the residing membrane to produce a midplane nano-bowl surface area of 700 nm2 and a projected in-plane area of 450 nm2. On the basis of the unique nano-bowl shape of the Piezo channel-membrane system, flattening the nonplanar TM blades may produce a maximal change of the projection area of ~250 nm2, generating energy to gate the channel (16). The curved configuration of the Piezo channels (PIEZO1 and PIEZO2) may further deform the membrane shape outside of the perimeter of the channel into a large, curved “membrane footprint” (48), which could further amplify the mechanosensitivity of the Piezo channel. Such “membrane-dome” (49) and “membrane footprint” (48) mechanisms have been proposed to account for the exquisite mechanosensitivity of PIEZO channels in response to various mechanical stimulation including poking and stretch forces. This would be an essential function of PIEZO1’s response to nonphysiological ultrasonic stimulation (0.5 MHz) (50).

We speculate that one of PIEZO2’s roles is to coordinate with hair-bundle mecanotransduction machinery to achieve ultrasonic transduction. Removal of either PIEZO2 or TMC1 abolished ultrasonic transduction (80 kHz) in cochlear hair cells (Fig. 5 D–G). This suggests that both PIEZO2 and hair-bundle mecanotransduction are required for ultrasonic transduction and explains why HEK293T cells expressing PIEZO2 fail to show ultrasonic response (SI Appendix, Fig. S8). PIEZO2 may be located at the apical surface of the OHCs, more specifically at the reticular lamina, where the stereocilia roots are least influenced by the membrane low-pass filtering. It cannot be excluded that PIEZO2 detects ultrasonic waves via adhesive junctions between OHCs and supporting cells (18) at the reticular lamina. In coordination with the vibration at the reticular lamina, which shakes the stereocilia from their roots, the hair-bundle mecanotransduction channels are activated reciprocally. Thus, it is possible that PIEZO2 itself may function only to detect ultrasonic waves rather than transducing them. We are intrigued with the idea that these data collected during auditory transduction may reflect functional consolidation of the two molecularly distinct mecanotransduction channels.
PIEZO2-mediated ultrasonic sensitivity in OHCs evolves over time. As Wu et al. proposed (18), the mechanosensitivity mediated by PIEZO2 and related Ca\(^{2+}\) modulation may support normal development of hair cells. However, in the first neonatal week, hair-bundle structure and function are still under development and are immature. This delay in maturation prevents the OHCs from accurately distinguishing the two PIEZO2 and mechanotransduction that subsequently un-masks PIEZO2-mediated mechanotransduction (18, 20).

On the other hand, the PIEZO2-mediated mechanotransduction may provide backup support for hair-bundle mechanotransduction (18), existing in both OHCs and IHCs. In mice at neonatal stage, PIEzo2 expression is found in both types of hair cells. After the onset of hearing, the two mechanotransduction channels establish their separate physiological specializations, respectively—hair-bundle–based machinery itself is enough for sonic transduction, while at ultrasonic range, PIEZO2 orchestrates hair-bundle mechanotransduction machinery supporting auditory transduction.

Interestingly, the cochlear OHCs, not the IHCs, are the effectors enabling PIEZO2 to sense ultrasound emissions (Fig. 4). This is the mechanism by which the animal gains extended spectral sensitivity from 16 kHz toward ultrasonic frequencies (Fig. 1F). Another OHC-specific protein is Prestin, a moiety that enhances cochlear mechanics and hearing sensitivity by providing OHCs somatic eM, which is not affected by PIEZO2 ablation. With PIEZO2, it is possible that the OHCs use a mechanism like ciliary motility to transfer ultrasonic vibration to the IHCs through the relative motion between the tectorial membrane and the reticular lamina. However, this putative mechanism needs further empirical evidence. The IHC may simply output the encoded ul-trasonic information from the organ of Corti. This is implied by empirical evidence. The IHC may simply output the encoded ultrasonic information from the organ of Corti. This is implied by empirical evidence.
A similar examination of Piezo2-null mice with ABR measurement was previously reported by Wu et al. (18) but in the lower frequency range. The authors suggest that mild differences (<10 dB) in ABR thresholds were observed in 12 to 28 kHz between Piezo2-CKO and Pax2f/f mice, which are similar with our results (Fig. 1F). However, the observation that Piezo2-CKO and Pax2f/f mice have similar ABR thresholds at 32 kHz seems to be in variance with our findings. Additionally, in our study, the control Pax2f/f mice had similar ABR thresholds with the B6 mice (Fig. 1 C and D), which is slightly different from their observation that the 2-mo Pax2f/f mice showed higher ABR thresholds (18).

Several factors may contribute to these differences. First, the two studies used different ABR electrode configurations. Using an electrode gently implanted in the skull in apposition to the brain, the mABR configuration was adapted to achieve higher stability and sensitivity. This is especially relevant for ABR signaling in the ultrasonic hearing range. Second, for ABR testing we used less developmentally mature 1-mo-old mice compared to the other study’s 2-mo-old cohort. Hearing sensitivity at different ages may vary slightly. Third, different genetic backgrounds for generating hybrid mice may result in shifts in their physiological functionality. Our data show that ultrasonic hearing is established, at minimum, from P21. Thus, the majority of our recordings were collected from 1-mo-old mice with their littermates used as controls. This was done to avoid the possibility of progressive deterioration that could result in erroneous data production.

In summary, we have discovered that PIEZO2 in OHCs plays an indispensable role in ultrasonic high-frequency hearing, suggesting an alternative auditory transduction mechanism for encoding frequencies in mammals. Given that both ultrasonic hearing and low-frequency hearing are conducted via cochlear OHCs but may be based on different mechanisms, it is of interest to investigate the response patterns to ultrasonic frequencies in cochlea, which is, whether such patterns follow the place-code principle of apical OHCs for low-frequency hearing, and very basal OHCs for ultrahigh-frequency hearing. It would be intriguing to study PIEZO2’s role in ultrasonic hearing from other species, for example, bats and whales. Furthermore, our study lays the foundation for addressing whether ultrasonic hearing and low-frequency hearing use distinct neural circuits and processing principles in brain regions along the ascending auditory pathways.

Materials and Methods

**Mouse Strains and Animal Care.** In this study, Cdh23f/f (stock no. 002552, named Cdh23-null in this study) B6.129-Tmct1(Eym)j (mice no. 019146, named Tmct1-KO in this study), and Rosa26 LSL H2B mcherry mice (stock no. 023139, named H2B-mcherry) were from the Jackson Laboratory (Bar Harbor, ME); Pax2-Cre mouse (named Pax2f/f in this study) was generated by Dr. Andrew Groves at Baylor College of Medicine (30) and Atoh1-Cre mice (named Atoh1f/f in this study) were kindly from Dr. Lin Gan in the Medical College of Georgia at Augusta University (31); Piezo2f/f (named Piezo2f/f in this study) were from the Jackson Laboratory (Bar Harbor, ME); and Piezo2-2f/f mice were gifts from Dr. Ardem Patapoutian at the Scripps Research Institute (26); Pmn-CreER (26) mouse line was a gift from Dr. Jian Zuo in School of Medicine at Creighton University (35); and vGlut3-P2A-ires-CreER knockin mouse strain was generated as described (37) and here named as vGlut3-CreER mouse. All the cKO mice were crossed in mixed genetic backgrounds for generating hybrid mice may result in shifts in their physiological functionality. Our data show that ultrasonic hearing is established, at minimum, from P21. Thus, the majority of our recordings were collected from 1-mo-old mice with their littermates used as controls. This was done to avoid the possibility of progressive deterioration that could result in erroneous data production. In summary, we have discovered that PIEZO2 in OHCs plays an indispensable role in ultrasonic high-frequency hearing, suggesting an alternative auditory transduction mechanism for encoding frequencies in mammals. Given that both ultrasonic hearing and low-frequency hearing are conducted via cochlear OHCs but may be based on different mechanisms, it is of interest to investigate the response patterns to ultrasonic frequencies in cochlea, which is, whether such patterns follow the place-code principle of apical OHCs for low-frequency hearing, and very basal OHCs for ultrahigh-frequency hearing. It would be intriguing to study PIEZO2’s role in ultrasonic hearing from other species, for example, bats and whales. Furthermore, our study lays the foundation for addressing whether ultrasonic hearing and low-frequency hearing use distinct neural circuits and processing principles in brain regions along the ascending auditory pathways.

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**Acoustic-Cue-Associated Freezing Behavior.** Male mice were used. Mouse location in an operant box (cubic, 30 × 30 × 30 cm) or an activity box (cylindrical, diameter of 35 cm and height of 30 cm) was monitored by an infrared camera with an infrared light as the light source, which was per-
treated in 0.5% PBST solution with 4% BSA at RT for 2 h with slow shaking. The tissues were incubated overnight at 4 °C with the primary antibody (Rabbit anti-GFP; 1:1500, Cat. A-11122, ThermoFisher Scientific) that was made in 0.5% PBST with 1% BSA and then washed with 0.1% PBST three times. Then, the cochlear tissues were incubated in the secondary antibody (Invitrogen anti-Rabbit Alexa Fluor 647, 1:1,000, Cat. A21244; Invitrogen Alexa Fluor 568 Phalloidin, 1:1,000, Cat. A12380) that was made in 0.5% PBST solution. Each incubation was shaken slowly.

Inner Ear Clarification. We used the polyethylene glycol (PEG)-associated solvent system (PEGASOS) method for inner ear clarification as previously reported (32). Mice were anesthetized by 0.4% pentobarbital sodium with an ip injection, followed by transcerebral perfusion with ice-cold 0.01 M PBS to wash out blood and then with 4% PFA. The inner ear was dissected in 4% PFA and fixed for 12 h at RT. After that, the following steps were performed in 37 °C shaker. The inner ear was immersed in 0.5M EDTA for 2 d with daily change for decalcification and ddH2O for 2 h to wash out remaining salt. Next, the inner ear was immersed in 25% Quadrol (diluted with H2O to a final concentration of 25% volvol, 122262, Sigma-Aldrich) for 2 d with daily change and 5% ammonium solution (diluted with H2O to a final concentration of 5% volvol, 105432, Sigma-Aldrich) for 6 h to decolorize. Then, ammonium solution was washed out with PBS for 30 min, followed by immunostaining steps. The inner ear was first immersed in blocking solution (4% BSA [V900933, VETEC] in 0.5% PBST) for 1 d, followed by Myo7a antibody (1:800, Rabbit, 25-6790, Protein Biosciences) in blocking solution for 2 d with daily change. Then, first antibody was washed out with 0.5% PBS-Triton X-100 for 1 d. Goat anti-Rabbit secondary antibody Alexa Fluor 488 (1:800, A11008, Thermofisher) in blocking solution was used to incubate for 2 d with daily change, also followed by washing out with PBS for 2 d. After that, de-lipidation was performed with the inner ear immersed in 30% tert-butanol (diluted with H2O, 360538, Sigma-Aldrich) for 4 h, 50% tert-butanol for 6 h, and 70% tert-butanol for 1 d. Then, the inner ear was immersed in tert-butanol (85%) (PEG) 70% vol/vol tert-Butanol, 27% vol/vol PEG methacrylate Mn 500 (PEGMMA500, 409529, Sigma-Aldrich) and 3% wt/vol Quadrol for 2 d with daily change for de-lipidation and BB-PEG (75% vol/vol benzyl benzoate [BB] [W213802, Sigma-Aldrich], 4% BSA [V900933, VETEC], and 3% wt/vol Quadrol) for 2 d with daily change for clearing. Clarified inner ear tissue was imaged with light-sheet microscope (Zeiss, Lightsheet Z.1) using 5× objective lens.

RNAscope Detection. After anesthesia with Avertin, transcerebral perfusion was done in P21 mice with ice-cold DEPC-PBS and then with 4% PFA (dilute from 16% PFA, DFO131, Leagene) in DEPC-PBS. Then, the temporal bones were dissected and fixed in fresh 4% PFA in DEPC-PBS at 4 °C for 12 h. After the postfixation, the cochlear tissue were decalcified by incubating at 120 mM EDTA decalcification solution at RT for 48 to 60 h. Then, the cochlear tissue were dehydrated in 15% sucrose solution in DEPC-PBS for about 30 min at 4 °C and in 30% sucrose solution in DEPC-PBS for about 2 h until the cochlear tissue sunk to the bottom of the tubes. After that, the tissue were embedded in O.C.T (optimal cutting temperature) (4583, Tissue-Tek) and stored at −80 °C. The tissues were sliced into 14-μm frozen sections (ThermoFisher Scientific) and stored at −20 °C no more than 8 h before RNAscope detection. The Pizoz2 transcript detection was performed according to manufacturer’s instructions using RNAscope detection kit S32100, ACDBio). Optimizing the procedure was time consuming because of difficulty keeping the temperature of tissue samples homogeneous, as shown by calibrated intensities covering the whole bottom of the recording dish (SI Appendix, Fig. S7).

Low-Frequency Fluid-Jet Stimulation to Hemicochlea. Fluid-jet configuration was used as previously reported (33). Briefly, a 35-mm diameter circular piezoelectric ceramic was sealed in a self-designed mineral oil tank. An electrical pulse of 5- to 10-μm diameter tip filled with recording solution (144 NaCl, 0.7 Na2PO4, 5.8 KCl, 1.3 CaCl2, 0.9 MgCl2, 10 H-Pepes, 5.6 D-Glucose in mM, pH 7.4, Osmolarity at 310 mmol/kg) was mounted into the tank and transmitted the pressure wave to the hair bundle of an OHC in hemicochlea samples. The circular piezoelectric ceramic driven by a sinusoidal voltage fluctuation generated from a patch-clamp amplifier (EPC10 USB, HEKA Elektronik) and amplified at 20-fold with a custom high-voltage amplifier. The 100-μs sinusoidal stimulation was given at frequency of 2,000 Hz and amplitude of 6.5 V.

Single-Cell Ca2+ Imaging and Whole-Cell Electrophysiology. HEK293T cells were plated onto 8-mm round glass coverslips, which were coated with poly-D-lysine and placed in 48-well plates. A total of 400 ng of plasmids were transiently transfected into HEK293T cells using lipofectamine 2000 (Life Technologies). The HEK293T cells were then cultured for 24 h to allow the HEK293T cells were imaged for Ca2+ signals by an upright microscope (BX51WI, Olympus) equipped with 60× water-immersion objective (LUMPlanFL, Olympus) and an sCMOS camera (ORCA Flash 4.0, Hamamatsu) controlled by MicroManager 1.6 software (52) with 50-ms exposure time and 1-s sampling interval. HEK293T cells were recorded using whole-cell patch clamp as previously described (54). All experiments were performed at RT (20 to 25 °C). Before the cell was transfected into a recording chamber with recording solution containing the following (in mM): 144 NaCl, 0.7 Na2PO4, 5.8 KCl, 1.3 CaCl2, 0.9 MgCl2, 5.6 glucose, and 10 H-Pepes (pH 7.4). The cells were imaged under an upright microscope (BX51WI, Olympus) with a 60× water-immersion objective and an sCMOS camera (ORCA Flash4.0, Hamamatsu) controlled by MicroManager 1.6 software (52). Patch pipettes were made from borosilicate glass capillaries (BF150-117 -10, Sutter Instrument Co.) with a pipette puller (P-9000, Sutter) and polished on a microforge (MF-830, Narishige) to resistances of 4 to 6 MΩ. Intracellular solution contained the following (in mM): 140 KCl, 0.1 EGTA, 2 Mg-ATP, 0.3 Na-GTP, and 10 H-Pepes, pH 7.2. The cells were recorded with a patch-clamp amplifier with a holding potential of −70 mV (EPC10 USB and PatchMaster software, HEKA Elektronik). The liquid junction potential is not corrected in the data shown. As measured, the pipette with CsCl intracellular solution had a value of +4 mV in regular recording solution.

Mechanical stimulation utilized a fire-polished glass pipette (tip diameter 3 to 4 mm) positioned at an angle of 80 relative to the cell being recorded as...
described (54). The probe was displaced by a piezoelectric actuator (P-601.1SL, Physik Instrumente) and driven by a piezoelectric crystal microstage (E625.01, Physik Instrumente). The probe velocity was 1 μm/s during the upward and downward movement, and the stimulus was kept constant for 100 ms. A series of mechanical steps in 1-μm increments was applied every 5 to 10 s.

NLC Recording. Neonatal mice at age of P7 to P8 were used. Basilar membrane with hair cells was dissected and bathed in external solution containing the following (in mM): 140 CsCl, 2 MgCl₂, 10 EGTA, and 10 H-Phes (pH 7.3 with NaOH; osmolality 300 mOsm with D-Glucose). The internal solution with the same pH and osmolality contained the following (in mM): 140 CsCl, 2 MgCl₂, 10 EGTA, and 10 H-Phes. Whole-cell patch clamp was done with holding potential at 0 mV (Axon Axopatch 200B, Molecular Devices Corp.). Continuous high-resolution two-site stimulus (390.6 and 781.2 Hz) with 10 mV peak amplitude superimposed onto a 250 ms voltage ramp (from +150 to −150 mV) was used. Data were acquired and analyzed using jClamp (Sicult). Capacitance-voltage data were fit with two-state Boltzmann function.

\[
C_m = \frac{2}{b + \exp \left( -\frac{e V_m - V_r}{k_B T} \right)} + C_{\text{lin}}
\]

where \(C_{\text{lin}}\) is linear membrane capacitance, \(Q_{\text{max}}\) is the maximum nonlinear charge, \(z\) is valence, \(e\) is electron charge, \(k_B\) is Boltzmann constant, \(T\) is absolute temperature, \(V_r\) is membrane potential, and \(V_m\) is voltage at peak capacitance.

Data Analysis. Each experiment contained at least three biological replicates. Data were managed and analyzed with MATLAB 2014b (MathWorks), MiniManager 1.6 software (52), Excel 2016 (Microsoft), Prism 6 (GraphPad Software), and Igor Pro-6 (WaveMetrics). All data are shown as mean ± SD, as indicated in the figure legends. We used a two-tailed t test for one-to-one comparison or one-way ANOVA for one-to-many comparison to determine statistical significance (*P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001). Nonparametric analysis was used accordingly if the data are not in Gaussian distribution or with equal variances. N numbers are indicated in the figures. For animal tracing and locomotion evaluation, videos of mouse locomotion in open-field, footshock, and pup-retrieval test were analyzed by MATLAB software and EthoVision XT software (version 11.5, Noldus). The center of the mouse was used to draw the locomotion trace. To show the speed information, the locomotion trace was dotted every 0.5 s. For footshock behavior analysis, freezing time percentage of precise (30 s before conditional stimulus) and postcue (30 s after conditional stimulus) were analyzed to compare the effect of sound-induced freezing. For Ca²⁺ data analysis, to extract fluorescence signals, we visually identified the regions of interest (ROIs) based on fluorescence intensity. To estimate fluorescence changes, the pixels in each specified ROI were averaged (F). Relative fluorescence changes, \(\Delta F/F_0\), were calculated as Ca²⁺ signal. The fluorescence imaging data were analyzed offline by MiniManager software and Excel software. The ROI was drawn to cover each hair cell. The fluorescence intensity of ROI was normalized to its value in the frame immediately prior to stimulation.

Data Availability. All study data are included in the article and/or supporting information.

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