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The sense of hearing is remarkable for its auditory dynamic range, which spans more than 10^12 in acoustic intensity. The mechanisms that enable the cochlea to transduce high sound levels without damage are of key interest, particularly with regard to the broad impact of industrial, military, and recreational auditory overstimulation on hearing disability. We show that ATP-gated ion channels assembled from P2X2 receptor subunits in the cochlea are necessary for the development of temporary threshold shift (TTS), evident in auditory brainstem response recordings as sound levels rise. In mice null for the P2RX2 gene (encoding the P2X2 receptor subunit), sustained 85-dB noise failed to elicit the TTS that wild-type (WT) mice developed. ATP released from the tissues of the cochlear partition with elevation of sound levels likely activates the broadly distributed P2X2 receptors on epithelial cells lining the endolymphatic compartment. This purinergic signaling is supported by significantly greater noise-induced suppression of distortion product otoacoustic emissions derived from outer hair cell transduction and decreased suprathreshold auditory brainstem response input/output gain in WT mice compared with P2RX2-null mice. At higher sound levels (≥95 dB), additional processes dominated TTS, and P2RX2-null mice were more vulnerable than WT mice to permanent hearing loss due to hair cell synapse disruption. P2RX2-null mice lacked ATP-gated conductance across the cochlear partition, including loss of ATP-gated inward current in hair cells. These data indicate that a significant component of TTS represents P2X2 receptor-dependent purinergic hearing adaptation that underpins the upper physiological range of hearing.

Sensory systems are characterized by adaptation processes that sustain transduction as stimulus intensity increases. The mammalian auditory system operates across an acoustic power range of ~120 dB, measured on the logarithmic decibel scale. The mechanism for the extraordinary acuity of the cochlea (recalling the age-old adage of “hearing a pin drop”) arises from the commitment of 75% of the sensory hair cells, the outer hair cells, to electromechanical (reverse) transduction, driving a “cochlear amplifier.” The nonlinear outer hair cell reverse transduction provides an ~40-dB gain at hearing threshold, reducing to zero as sound levels rise (1).

A major challenge for auditory physiology is to understand how hearing is preserved in the face of acoustic overstimulation, as noise can damage the cochlea and can greatly exacerbate hearing loss with aging (2). Given the recent propensity for direct delivery of high-level recreational sound to the ear canals by personal music players and, more broadly, the impact on our hearing of noise from industrial and military environments, there is an imperative to better understand the intrinsic mechanisms that enable the cochlea to accommodate loud sound.

Known mechanisms by which the cochlea adjusts its sensitivity to loud sound include the middle-ear muscle reflex and efferent feedback to the outer hair cells. The middle-ear muscle reflex (3) is largely driven by vocalization or intense low-frequency sound, and fatigues after a few minutes. Efferent neuronal adaptation is even more rapid (milliseconds to seconds) and provides dynamic modulation that enables the cochlea to mask sounds of attentional interest from background noise. The olivocochlear efferent system is to some extent otoprotective against noise damage (4), and contributes to “conditioning,” where sustained moderate sound exposure toughens the cochlea against subsequent acoustic overstimulation (5). However, this efferent feedback to the cochlea rapidly adapts at sound levels well below safe upper hearing limits (85 dB LAeq [equivalent continuous A-weighted sound pressure level (dB)], as reflected in workplace legislation (6). In this study, we investigated the hypothesis that purinergic signaling contributes to cochlear adaptation to elevated sound levels and protection from overstimulation. A complementary report by Yan et al. (7) shows that a dominant-negative point mutation in the ATP-gated ion channel P2X2 receptor subunit underlies the autosomal-dominant nonsyndromic progressive hearing loss locus DFNA4. This study included data using the P2X2 receptor-encoding gene knockout (P2RX2-null) mouse model, which demonstrated that noise exposure over a significant fraction of the animals’ lifetime caused selective high-frequency hearing loss.

The P2X2 receptor is abundantly expressed by cells lining the cochlear partition, including the sensory hair cells of the organ of Corti, Reissner’s membrane epithelial cells, and spiral ganglion neurons (8–10). The cochlear partition maintains the positive endocochlear potential (EP; ~140 mV) that, along with the negative membrane potential of the hair cells, provides the driving force for sound transduction (11). Both EP and the hair cell membrane potential are reduced by activation of P2X2-like ATP-gated nonselective cation channels (12, 13). In the guinea pig cochlea, noise stress causes release of ATP into the K+–rich endolymphatic compartment, where P2X2 receptors are concentrated (14). Thus, a role for ATP regulation of cochlear


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function via P2X2 receptors is well-established at the cellular and tissue level, but analysis of the potential contribution of this pathway to the regulation of hearing has previously lacked an appropriate animal model. Here we provide evidence that as the sound floor is elevated, ATP is released into the cochlear partition, activating P2X2 receptors, which reduces sound transduction and synaptic transmission from the hair cells. This purinergic regulation of hearing sensitivity was revealed by the absence of temporary threshold shift (TTS) in P2RX2-null mice. The mechanism is otoprotective, as P2RX2-null mice are highly vulnerable to noise-induced hearing loss with more extensive acoustic overstimulation.

**Moderate Noise TTS Is P2X2 Receptor-Dependent**

Auditory brainstem response (ABR) thresholds to tone pips (4–32 kHz) were initially compared between P2RX2-null mice and background wild-type (WT) strain (C57BL/6J) mice at 6 mo of age. Baseline thresholds were comparable (Fig. S1). Remarkably, the P2RX2-null mice lacked the pronounced increase in threshold (loss of hearing sensitivity) to a moderately high, closed-field noise exposure [30 min; 85 decibel sound pressure level (dB SPL); 8–16 kHz band-pass noise] evident in the WT controls when measured using click and 16-kHz tone pip stimuli within ~30 min of the noise exposure (Fig. 1A and B). The difference in mean thresholds post-versus pre-noise in WT mice was 15.6 ± 4.8 dB (P = 0.001) compared with 1.4 ± 2.6 dB (P = 0.596) in P2RX2-null mice.

Cubic (2f1-f2) distortion product otoacoustic emissions (DPOAEs) recorded alongside ABR testing (before noise and ~30 min after noise), were used to assess outer hair cell function and cochlear micromechanics before and after noise exposure in P2RX2-null and WT mice. In the 6-mo-old WT group, the 30-min 85-dB octave band noise exposure significantly elevated the thresholds of DPOAEs arising from primary tones about 16 kHz (P = 0.009, paired t test; 19.4 ± 4.3 dB prenoise; 37.5 ± 5.0 dB postnoise; n = 8; average threshold shift 18.1 ± 5.1 dB), whereas DPOAE thresholds in P2RX2-null mice were not affected by the noise exposure (P = 1.000, Wilcoxon signed-rank test; n = 7; 25.5 ± 4.2 dB prenoise, 33.6 ± 4.3 dB postnoise; average threshold shift 7.9 ± 2.6 dB) (Fig. S2). DPOAEs reflect the contribution of the outer hair cell-derived cochlear amplifier to hearing sensitivity and frequency selectivity (15), and these data indicate that noise-induced P2X2 receptor activation inhibits outer hair cell electromotility and affects cochlear micromechanics. This suppression of the cochlear amplifier would contribute to the elevation in thresholds (TTS) evident from the ABR recordings in WT animals. To further evaluate this, we assessed the noise-induced (closed-field, 85 dB, 8–16 kHz octave band noise) modulation of the operating point of the cochlear amplifier by measuring the change in amplitude of the cubic DPOAE about 16 kHz using 60-dB primary tones in WT mice (n = 4) compared with P2RX2-null (n = 4) littermates (3-mo-old). Both groups of mice had equivalent starting DPOAE amplitudes (WT 20.3 ± 1.8 dB SPL; P2RX2-null 19.7 ± 0.8 dB SPL; P = 0.231, t test). However, following 1–2.5 min of noise, P2RX2-null mice exhibited significantly less reduction in DPOAE amplitude than WT mice (P < 0.05; two-way repeated-measures ranked ANOVA; Fig. S3). The reduction in DPOAE amplitude changed from ~34.5 ± 1.6 dB to ~28.5 ± 2.8 dB over 45 min in WT mice, compared with a recovery from ~27.0 ± 2.9 dB to ~19.8 ± 4.1 dB in P2RX2-null mice.

The time course for the development of the P2X2 receptor-dependent TTS was determined by successive 16-kHz tone pip ABR threshold measurements after 10, 40, and 110 min of noise exposure (closed-field, 4–32 kHz noise band, 85 dB SPL). The study used P2RX2-null mice and strain-matched WT controls (9–12 wk of age; n = 3 for each group). The WT mice showed rapid development of TTS (Fig. 2A and Fig. S4), which was significantly greater than the minimal change in thresholds in the P2RX2-null mice over the 2-h study period (P = 0.002, two-way ANOVA). ABR thresholds during noise exposure were subtracted from baseline threshold values prenoise (t0) to provide the TTS for each mouse. These data were fitted to a single exponential growth function [f = 17.8 × (1 − exp[−0.0514 × T(min)])]; R² = 0.942] to yield a time constant of ~20 min for the development of TTS, with an asymptote of ~18 dB (Fig. 2A). In contrast, P2RX2-null mice had minimal TTS, with an ~6 dB asymptote [P < 0.001, two-way ANOVA versus WT group; P2RX2-null best fit: f = 6.035 × (1 − exp[−0.0352 × T(min)]); R² = 0.988].

The time course for recovery from P2X2 receptor-dependent TTS was determined by measuring the ABR thresholds in WT, heterozygous [P2RX2(−/−)], and homozygous knockout (P2RX2-null) littermates (8–12 wk; n = 6 per group) after 30 min of broadband noise (~TTS asymptote) (closed-field, 4–32 kHz, 85 dB SPL). The starting level of TTS (time 0; Fig. 2B) in the WT mice was 15.4 ± 2.2 dB, which was not significantly different from the TTS in the heterozygous P2RX2(−/−) mice (17.5 ± 3.8 dB; P = 0.929, one-way ANOVA with Holm–Sidak pairwise comparison). Predictably, P2RX2-null mice had no significant threshold shift (1.7 ± 0.8 dB; P = 0.102, one-sample t test). The difference between the P2RX2-null group and either WT or P2RX2(−/−) mice was highly significant (P < 0.001). These data indicate haplosufficiency in the development of P2X2 receptor-dependent TTS. The ABR input/output functions were determined pre- and postnoise for the WT and P2RX2-null mice based on the amplitude of the p1-n1 wave (Fig. S5). These data indicate a significant noise-induced reduction in the gain of sound transduction in WT mice (P < 0.001, two-way ranked ANOVA) but not in P2RX2-null mice. There was no significant difference between the growth functions for the WT and P2RX2-null mice before noise. ABR thresholds were remeasured at 8, 24, and 96 h postnoise. The time course of the TTS in the P2RX2-null mice had a time constant of 12.3 h [single exponential decay best fit; f = 1.70 + 13.44 × exp(−0.0814 × T(h)); R² = 0.98]. The recovery from TTS in the P2RX2(−/−) mice mapped to the WT recovery time course. These data indicate that 30 min of noise activation of P2X2 receptor signaling (in WT mice) instigates a prolonged reduction in hearing sensitivity that takes more than 24 h to fully resolve.

![Fig. 1.](image-url) Fig. 1. P2RX2-null mice (KO) lacked TTS in response to moderate-intensity noise evident in WT controls. (A) Examples of ABR traces (10-ms duration) for click stimuli before and after 30-min noise (85 dB, closed-field). The threshold in the WT mouse increased from 32.5 to 45 dB (blue bar), whereas the P2RX2-null mouse threshold remained unchanged (red bar). (B) Box plots with data overlay showing the p1-n1 wave in WT mice had a time constant of 12.3 h [single exponential decay best fit; f = 1.70 + 13.44 × exp(−0.0814 × T(h)); R² = 0.98]. The recovery from TTS in the P2RX2(−/−) mice mapped to the WT recovery time course. These data indicate that 30 min of noise activation of P2X2 receptor signaling (in WT mice) instigates a prolonged reduction in hearing sensitivity that takes more than 24 h to fully resolve.
P2RX2-Null Mice Have Greater Hearing Loss in High-Level Noise

We titrated the noise level to determine the contribution of cochlear P2X2 receptor signaling to TTS and persistent threshold shift (PTS) at higher sound levels, levels that would be expected to induce more extensive TTS, but no PTS, in WT mice. Noise at 95 dB SPL for 30 min (8–16 kHz octave band, closed-field) caused substantial immediate threshold shifts in strain-matched WT mice (n = 11) (≥45 dB from 16 kHz) as well as P2RX2-null mice (n = 11) (10- to 13-wk-old) (Fig. 3A). Thus, P2X2 receptor-dependent TTS was overshadowed by additional components at this noise level (no significant difference between genotypes, P = 0.206, two-way ANOVA, 4 kHz). Reassessment of ABR in both groups of mice 2 wk later indicated that the threshold shifts measured immediately after noise across the test frequencies in the WT mice were all TTS, whereas in the P2RX2-null mice there was a substantial (~20 dB) PTS at the highest frequency tested (24 kHz) (P < 0.001, one-sample t test). This transfer of the impact of loud sound to higher-frequency transducing regions of the cochlea is consistent with the well-established half-octave shift in the frequency of maximum loss that reflects the underlying cochlear amplifier energy delivery (16). This PTS was associated with a significantly greater immediate DPOAE threshold shift in P2RX2-null mice compared with WT (Fig. S6; P = 0.026, 16–28 kHz, two-way ANOVA), suggesting that in the absence of P2X2 receptor activation at this high noise level, outer hair cells located basal to the tonotopic place for the noise band are overdriven.

The effects of sound levels that would be expected to induce PTS in WT mice were then assessed using open-field noise (2 h, 100 dB SPL, 8–16 kHz band-pass noise) in 3-mo-old P2RX2-null mice and strain-matched WT controls. Baseline ABR thresholds (click and tone pip stimuli) were again equivalent in P2RX2-null and WT mice before noise exposure. However, PTS assessed 2 wks after noise exposure was substantially and significantly higher in P2RX2-null mice. For click stimuli (Fig. 3B), the PTS in the P2RX2-null group was 18 dB greater than that of the WT controls (P = 0.026, Mann–Whitney rank-sum test; n = 9 P2RX2-null; n = 8 WT). Similarly, PTS at or above the frequency of the octave band noise was substantially (up to 32 dB) greater than that of the WT (P < 0.001, two-way ANOVA, Holm–Sidák pairwise comparison; Fig. 3B).

Histological analysis of the cochlear tissue from these 100 dB SPL noise-exposed mice at 6 wks postnoise, using toluidine blue-stained thin sections following resin embedding, indicated that there was significant (P = 0.008, two-tailed unpaired t test) atrophy of the spiral ganglion neurons in P2RX2-null mice (Fig. 3C and Fig. S7 A, B, and E). Somata size in the midcochlear region decreased from 118.9 ± 3.6 μm² (from averages of five WT cochleae) to 95.2 ± 5.8 μm² (from averages of four P2RX2-null cochleae) (95–156 neurons measured per cochlea). There was, however, no significant difference in neuron density (WT 32.9 ± 2.5, P2RX2-null 37.6 ± 5.6 neurons/10,000 μm²; P = 0.345) that would indicate neuronal loss (Fig. S7E).

Fig. 2. TTS development and recovery. (A) Development of TTS was determined using 16-kHz ABR threshold measurements by briefly interrupting sustained noise (85 dB, 4–32 kHz, closed-field; K/XA anesthesia); knockout (P2RX2-null) (n = 3) mice; wild-type (n = 3) age- and strain-matched control mice. Single exponential curve fits for TTS growth; time constant 19.5 min WT; 28.4 min KO. P < 0.001 KO vs. WT; **P < 0.003, ***P < 0.001, two-way ANOVA, Holm–Sidák pairwise comparison. (B) Recovery of TTS in WT, HET (P2RX2+/-), and KO littermate mice using broadband noise (4–32 kHz, 30 min, closed-field; n = 6 each group). ABR (16-kHz) threshold measurements. P < 0.001 for KO vs. WT or HET, two-way ANOVA. TTS recovery time constant for WT or HET was 12.3 h.

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plexus that envelopes the base of the inner hair cells (neurofilament 200 immunolabeling; Fig. 3E). These data indicate a direct effect on hair cell synapses and neurons, likely via glutamatergic excitotoxicity (17). Data from control experiments, where wild-type mice were exposed to a noise level known not to produce PTS (95 dB noise, 8–16 kHz, closed-field, 30 min, as for Fig. 2A), showed that there was no difference in CtBP2 puncta per inner hair cell between left (untreated) versus right (noise-exposed) cochlea (noise: 17.0 ± 2.6; no noise: 19.0 ± 2.7; P = 0.558, paired t test; n = 5). These data indicate that unlike the noise-exposed cochlear susceptible to PTS due to loss of P2X2 receptor signaling, decreased synaptic density does not occur in wild-type mice exposed to loud noise insufficient to drive PTS with P2X2 receptor signaling in place. Noise-exposed wild-type mice with P2RX2- are known to exhibit decreased CtBP2 labeling (18).

Sites of Action

Immunofluorescence confirmed the cochlear partition as the principal site of P2X2 receptor expression in WT mice (Fig. 4A, Fig. S8, and Movie S1). P2X2 receptor immunolabeling included all of the cells lining the endolymphatic compartment with the exception of the marginal cells of the stria vascularis. In contrast, P2X2 receptor immunolabeling in the spiral ganglion was minimal. This mouse cochlear P2X2 receptor distribution matched the P2X2 receptor mRNA transcript expression in the rat cochlea detected by in situ hybridization (8). Specificity of the immunolabeling was confirmed by the absence of signal in P2RX2-null cochlear tissue.

The cellular physiology of the cochlear partition was investigated by patch-clamp analysis of Reissner’s membrane epithelial cells as well as the inner and outer hair cells. These cell types have previously been shown to have substantial ATP-gated nonselective cation conductances localized to the endolymphatic surface in guinea pig (9, 10, 19, 20), rat (21), and mouse (22). In no case was an ATP-activated inward current recorded from P2RX2-null Reissner’s membrane epithelial cells (n = 30), inner hair cells (n = 15), or outer hair cells (n = 6). In contrast, in WT tissue, Reissner’s membrane epithelial cells had a mean ATP-activated inward current of −2.06 ± 0.17 nA; n = 63/63 cells responded; outer hair cells had a mean inward current of −312 ± 78 pA; n = 77 cells; and inner hair cells had a mean inward current of 304 ± 210 pA (3/16 cells responded to ATP) (Fig. 4B and C). Of note was the approximately sevenfold larger ATP-gated inward currents in the Reissner’s membrane cells compared with the hair cells. The substantial Reissner’s membrane ATP-activated conductance indicates that this element of the cochlear partition would contribute significantly to the overall ATP-induced reduction in cochlear partition resistance (CoPR) evident when ATP is introduced into the endolymphatic compartment (Discussion). There were no significant differences in resting membrane potential, measured at zero current, or variations in voltage-dependent conductance, between P2RX2-null and WT for any of the cell types (Table S1) to indicate that the absence of the P2X2 receptor had any bearing on the properties of the cells other than the loss of the capability for ATP-gated inward current.

The effect of ATP injection into the endolymphatic compartment on the EP and CoPR was assessed in vivo to establish whether additional ATP-activated conductance remained in the absence of P2X2 expression in the cochlear partition cell types tested by patch-clamp. As established in the guinea pig model (12), microinjection of ATP into scala media in WT mice produced a significant fall in EP and a corresponding reduction in CoPR (Fig. 4D and E). The EP directly impacts on the driving force for sound transduction (11). P2RX2-null mice failed to show any change in EP or CoPR when ATP [100 μM (n = 5) or 1 mM (n = 7)] (n = 12 baseline measurements) was injected (10 nL) into scala media (P = 0.009, two-way ranked ANOVA compared with WT). WT mice showed a dose-dependent reduction in EP [15% at 100 μM ATP (P < 0.001, one-way ranked ANOVA [n = 13] vs. baseline [n = 25]) and 37% at 1 mM ATP (P = 0.001 vs. 100 μM ATP, paired t test; n = 12)]. The baseline EP was similar in both in both WT and P2RX2-null mice (WT: 110.0 ± 4.0 mV; P2RX2-null: P = 0.154, unpaired t test). The ATP-induced fall in EP in WT mice reflects the capacity of the cells lining scala media to shunt K+ across the cochlear partition via the ATP-gated nonselective cation channels (12). The increase in conductance in these cells is evident in the associated dose-dependent reduction in CoPR in the WT mice (P < 0.001 baseline vs. 100 μM and 1 mM ATP; one-way ANOVA). There was no change in CoPR with ATP injection in P2RX2-null mice (P < 0.001 compared with WT, two-way ANOVA), whereas baseline resistance was not significantly different from that of the WT mice (WT: 6.71 ± 0.10 kΩ; P2RX2-null: 6.91 ± 0.16 kΩ; P = 0.236). Given that the endolymph volume of the mouse cochlea is ~0.2 μL (23), we estimate −5–50 μM ATP concentrations arising from the 10-nL injections, which approaches the EC50 for ATP-gated ion channels assembled from P2X2 receptor subunits (24).

These data indicate that in P2RX2-null mice there is no detectable compensation due to up-regulation of other P2X receptor subtypes (indicating homomeric P2X2 receptor subunit assembly of the ATP-gated ion channels) across the range of epithelial cells shown to express P2X2 receptors (Fig. 4A and Movie S8). In addition, these data, and the normal hearing function of P2RX2-null mice in the absence of noise stimulation, make it unlikely that critical molecular pathways supporting sound transduction and synaptic transmission have been affected by the knockout of the P2RX2 gene. Noise-induced ATP release would therefore be unable to invoke cation conductance in the sensory epithelium and other elements of the cochlear partition that control sound transduction in P2RX2-null mice.

**Discussion**

P2RX2-null mice failed to exhibit the initial 15 dB of threshold shift produced when sound level was elevated. WT mice developed this TTS with a 20-min time constant. The impact of moderate noise on cochlear function was also reflected in the substantial decrease in suprathreshold ABR amplitude in WT mice compared with the preserved gain of the ABR input/output response following exposure to the same noise level (8, 13, 14). Importantly, the absence of P2X2 receptors in the cochlea diminished noise-induced shifts in threshold. These results indicate a potential role for P2X2 receptors in the maintenance of normal cochlear function and in its susceptibility to noise-induced injury.

The localization of P2X2 receptors reflects the capacity of the sensory epithelium to shunt K+ across the cochlear partition via the ATP-gated nonselective cation channels (12). The increase in conductance in these cells is evident in the associated dose-dependent reduction in CoPR in the WT mice (P < 0.001 baseline vs. 100 μM and 1 mM ATP; one-way ANOVA). There was no change in CoPR with ATP injection in P2RX2-null mice (P < 0.001 compared with WT, two-way ANOVA), whereas baseline resistance was not significantly different from that of the WT mice (WT: 6.71 ± 0.10 kΩ; P2RX2-null: 6.91 ± 0.16 kΩ; P = 0.236). Given that the endolymph volume of the mouse cochlea is ~0.2 μL (23), we estimate −5–50 μM ATP concentrations arising from the 10-nL injections, which approaches the EC50 for ATP-gated ion channels assembled from P2X2 receptor subunits (24).

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function of P2RX2-null mice. Recovery from the P2X2 receptor-dependent TTS had a 12-h time constant, which is similar to the situation with human subjects exposed to TTS-level sound (25). In the absence of P2X2 receptor signaling, the cochlea was more vulnerable to damage from excessive noise, evident as PTS in the P2RX2-null mice at the higher presented sound levels. Given that perceived loudness halves every 10 dB and sound intensity halves every 3 dB, this P2X2 receptor-dependent hearing modulation provides a substantial downward adjustment of the operational point of sound transduction and auditory neurotransmission in the cochlea at high sustained sound levels. These characteristics provide strong support for the postulate that sound-induced activation of P2X2 receptors in the cochlea underlies a local autocrine/paracrine purinergic adaptation mechanism that sustains the upper physiological range of hearing.

Although it is appreciated that TTS (reversible within hours to days) and PTS are not a continuum of cochlear pathophysiology due to acoustic overstimulation, noise-induced threshold changes are multifactorial, as revealed by histological, physiological, proteomic, and transcriptional studies of the cellular and molecular responses of the cochlea to loud sound (e.g., 17, 25–29). The mechanisms underlying TTS have remained speculative, although studies suggest that oxidative stress at the inner hair cell afferent synapse is significant (30). Even low-level noise stress (producing PTS with sound exposure) induces decreases for weeks — evident in earlier development of hearing loss with aging (31).

The identification that purinergic signaling drives TTS as the background sound level rises is key evidence that such TTS is fundamentally adaptive. Neural regulation via the olivocochlear efferent innervation to the outer hair cells can certainly protect the cochlea from noise-induced hearing loss (5, 32), but the kinetics for efferent regulation of cochlear function, such as contralateral supression via the medial olivocochlear bundle, have fast time constants (4) inconsistent with TTS. The most parsimonious explanation arising from our study is that P2X2 receptor activation, as a result of noise-induced ATP release in the cochlea, represents the primary upstream signaling element causing the dominant component of TTS at moderate sustained noise levels, and this reflects physiological adaptation to noise rather than an injury response. Certainly, the preservation of hearing sensitivity in the face of sustained 85-dB noise exposure in P2RX2-null mice attests to the lack of immediate tissue damage as an underlying cause of the TTS in WT mice at this sound level. This adaptation process would enable cochlear hair cells to modulate the upper component of the physiological hearing range in batch by noise with minimal changes for weeks. A potential advantage of our results is that the P2RX2-null mice lacked P2X2 receptor expression in the cochlea from early development. However, underlying hearing physiology was normal (Fig. S1; see also ref. 7). Older P2RX2-null mice do exhibit selective hearing loss, evident as exacerbated presbycusis compared with wild-type controls at 18 mo of age raised in the quiet (7).

The mechanism of the P2X2 receptor-dependent reduction in hearing sensitivity surely involves the movement of cations (including a high Ca2+ conductance) through P2X2 receptors homomeric ATP-gated channels (24). At a tissue level, it has been shown that release of ATP into the endolympathic compartment causes a rapid shunt of K+ entering the ATP-gated ion channels (12) (confirmed here as exclusively P2X2 receptor-mediated), which, via the associated fall in EP, and parallel depolarization of the hair cells, would contribute to regulation of sound transduction. However, extracellular ATP is rapidly hydrolyzed by ecto-ATPases (33). Because TTS arising from 30 min of 85-dB noise persisted for hours, well beyond the likely period of elevated extracellular ATP, a more sustained effect must underlie P2X2 receptor-dependent TTS. A clue may be found in the observation that outer hair cell electromotility (reverse transduction) in WT mice was compromised with PTS (DPOAE threshold was elevated and DPOAE amplitude was suppressed; Figs. S2 and S3), alongside the increase in ABR threshold (decreased neural output). EP recovers rapidly after cessation of noise, and direct action of K+ efflux is also unlikely to account for the TTS, as clearance of sound-induced K+ buildup around the inner hair cells is too rapid (34). The hair cell transducer conductance (forward transduction) is relatively resistant to acoustic overstimulation, where, despite considerable increases in cochlear nerve thresholds (TTS), cochlear microphonic output (a population measure of outer hair cell receptor potential) is sustained (35). Based on reduced tuning of basilar membrane displacement associated with this TTS, it has been proposed that outer hair cell reverse transduction (rather than forward transduction) was affected (35). Ca2+-mediated adaptation affecting outer hair cell electromotility (36) and supporting cell micromechanical compliance, as well as Ca2+-regulated adaptation at the inner hair cell ribbon synapse (37, 38), are all processes likely to be impacted by the distributed cochlear P2X2 receptor signaling.

Our findings generate an apparent paradox in that moderate noise fails to affect hearing in P2RX2-null mice (speaking to the robust nature of forward and reverse transduction without P2X2 receptor-dependent adaptation) but loud noise causes enhanced PTS in these animals. This dichotomy in sensitivity to noise in P2RX2-null mice can be reconciled if the TTS-level adaptation arising from noise-activated P2X2 receptor signaling protects transduction and transmission at the hair cells from excessive acoustic stimulation, whereas PTS, focused at the inner hair cell–spiral ganglion synapse, arises from overdrive of these processes in the absence of this purinergic adaptation mechanism. Noise-induced glutamate excitotoxicity is well-established in the cochlea (17, 27), and it is correlated with loss of the spiral ganglion punctate synapses, atrophy of the neuron soma, and, presynaptically, loss and dislocation of the CtBP2-labeled ribbon synapses (18). All of these characteristics match the cochlear structural profile of P2RX2-null mice with PTS.

The identification of the P2X2 receptor as a key element of hearing adaptation suggests that factors that compromise this process, such as age-related muting of P2X2 receptor-dependent regulation of cochlear partition conductance (39), or polymorphisms within the P2RX2 gene, contribute to variation in susceptibility to noise-induced hearing loss in society. Indeed, the present study clearly complements our recent report (7), which showed that the absence of cochlear P2X2 receptor signaling in two Chinese families, attributable to a dominant-negative mutation P2RX2 c.178G>T (p.V60L), removed intrinsic purinergic otoprotection from the hearing organ and precipitated the DFNA41 autosomal-dominant progressive hearing loss. DFNA41 subjects with a history of noise exposure had exacerbated high-frequency hearing loss, an effect that was modeled in P2RX2-null mice exposed to long-term noise exposure. In identifying a key molecular element of hearing adaptation to sustained noise stressors, the present study provides a timely reinforcement of the consequence of exceeding the capacity of our hearing organ to cope with loud sound. These experiments open a path for analysis of the mechanisms for sound-evoked ATP release into the cochlear tissues associated with this P2X2 receptor signa
ing, including potential contributions from P2Y receptors linked to ATP release (40), and cellular elements that sustain dynamic adap-
tation to changes in sound level over a lifetime.
Affairs Healthcare System, and the University of New South Wales Animal Care and Ethics Committee. The mice were maintained in individually ventilated cages (VentriRack; BioZone).

ABR and DPOAE. Sound levels are presented as dB sound pressure level (SPL). Click and puretone ABR thresholds and cubic DPOAEs (2f2−f1) were measured under ketamine/xylazine/acepromazine (K/X/A) anesthesia as previously described (43). P2X2 receptor-dependent TTS was produced using 85 dB SPL noise (8–16 kHz or 4–32 kHz), delivered via the ear probe (closed-field). PTF was generated in conscious mice using 2·100-dB SPL open-field broadband noise (8–16 kHz band-pass), or under K/X/A anesthesia (4–32 kHz, 100 dB SPL, 1 h, closed-field).

Endocochlear Potential and Cochlear Partition Resistance. EP and CoPR measurements were made under urethane anesthesia as previously described (12), with the minor modification of a 500-ms 1-µA current pulse at 50% duty cycle.

Cellular Physiology. Whole-cell patch-clamp recordings from Reissner’s membrane epithelial cells and hair cells were made in situ as previously described (22). Cells were voltage-clamped at −60 mV and ATP pressure applied to the cells via a glass micropipette.

33. Cockayne DA, et al. (2005) P2X2 knockout mice and P2X2/P2X3 double knockout mice reveal a role for the P2X2 receptor subunit in mediating multiple sensory effects of ATP. J Pharmacol Exp Ther 312(3):621-630.

Immunofluorimetry. Immunofluorescence imaging of P2X2 receptor expression was measured using an anti-P2X2 receptor polyclonal antisera (Alomone). CTBP2 immunofluorescence of the hair cell ribbon synapses used an anti-CTBP2 polyclonal antibody (BD Biosciences). Neurofloumint 200 immunofluorimetry of spiral ganglion neurites projections to the hair cell synapses was measured using an anti-neurofloumint 200 antibody (Sigma). After mounting, the immunofluorimetry was imaged using a Zeiss 710 confocal microscope.

Data Analysis. Parametric and nonparametric data comparisons were performed using two-way ANOVA; Student t tests, Mann-Whitney rank-sum tests, and Wilcoxon signed-rank tests after assessment of normality and variance (Sigmastat version 11; Systest Software). Threshold for significance α = 0.05, with Holm–Sidak all pairwise multiple comparison, where appropriate. Data are presented with SE (SEM).

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