Onset kinetics of noise-induced purinergic adaptation of the ‘cochlear amplifier’

Jennie M. E. Cederholm¹ · Allen F. Ryan^{2,3} · Gary D. Housley¹

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Abstract

A major component of slowly reversible hearing loss which develops with sustained exposure to noise has been attributed to release of ATP in the cochlea activating P2X₂ receptor (P2X₂R) type ATP-gated ion channels. This purinergic humoral adaptation is thought to enable the highly sensitive hearing organ to maintain function with loud sound, protecting the ear from acoustic overstimulation. In the study that established this hearing adaptation mechanism as reported by Housley et al. (Proc Natl Acad Sci U S A 110:7494–7499, 2013), the activation kinetics were determined in mice from auditory brainstem response (ABR) threshold shifts with sustained noise presentation at time points beyond 10 min. The present study was designed to achieve finer resolution of the onset kinetics of purinergic hearing adaptation, and included the use of cubic (2f₁–f₂) distortion product otoacoustic emissions (DPOAEs) to probe whether the active mechanical outer hair cell ‘cochlear amplifier’ contributed to this process. We show that the ABR and DPOAE threshold shifts were largely complete within the first 7.5 min of moderate broadband noise (85 dB SPL) in wildtype C57Bl/6J mice. The ABR and DPOAE adaptation rates were both best fitted by a single exponential function with ~3 min time constants. ABR and DPOAE threshold shifts with this noise were minimal in mice null for the *P2rx2* gene encoding the P2X₂R. The findings demonstrate a considerably faster purinergic hearing adaptation to noise than previously appreciated. Moreover, they strongly implicate the outer hair cell as the site of action, as the DPOAEs stem from active cochlear electromotility.

Keywords Distortion product otoacoustic emission · P2X₂ receptor · Auditory brainstem response · Noise-induced hearing loss · Temporary threshold shift · Mouse

Introduction

Our hearing normally accommodates a dynamic soundscape that ranges from very low to high intensities. However, acoustic overstimulation from a range of environmental and occupational sources is accelerating hearing loss during our lives [1].

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✉ Gary D. Housley
g.housley@unsw.edu.au

¹ Translational Neuroscience Facility and Department of Physiology, School of Medical Sciences, UNSW Sydney, Sydney, NSW, Australia

² Departments of Surgery and Neurosciences, University of California San Diego, La Jolla, CA, USA

³ Veterans Administration Medical Center, La Jolla, CA, USA

Recreational music particularly impacts on the hearing of young people [2, 3]. Despite acute awareness of the human and corporate costs of loss of hearing due to noise exposure, and broad workplace health and safety regulatory controls, noise-induced hearing loss (NIHL) presents an apparently intractable challenge. For example, the primary basis for unscheduled discharge from the US Military is NIHL [4]. As a result, the on-going costs of veteran support for NIHL and associated tinnitus represent the largest cost factor in disability payments by the US Veterans Health Administration. Treatment of hearing loss is problematic as the hair cells that transduce sound and the spiral ganglion neurons that transmit auditory information to the CNS cannot be regenerated and there are currently no effective treatments outside use of auditory prostheses. Improved understanding of intrinsic mechanisms that protect the cochlea from NIHL may facilitate development of novel hearing therapeutics.

Acoustic overstimulation, depending upon frequency, level and duration, leads to a continuum of temporary or permanent

sensorineural hearing loss [5–7]. The mechanisms underlying development of reversible noise-induced changes in our hearing sensitivity, which are intrinsically protective, involve a range of cochlear elements, including the ossicular chain middle ear reflex and inhibitory olivocochlear efferent feedback to the outer hair cells [8]. A major additional mechanism is purinergic hearing adaptation mediated by acoustically induced release of ATP in the cochlea that activates $P2X_2$ receptors ($P2X_2R$). These receptors, which assemble as trimeric ATP-gated non-selective cation channels, are broadly expressed in the cochlea, with prominent expression by cochlear partition epithelial cells, including the organ of Corti (Fig. 1), inner and outer sulcus and Reissner's membrane [9–11]. Spiral ganglion neurons also express $P2X_2R$, alongside other $P2X$ subtypes [12–14]. $P2X_2R$ expression is upregulated in both the spiral ganglion and cochlear duct with noise exposure; where rat outer hair cells demonstrated > 3-fold increase in ATP-gated inward currents [12]. We previously demonstrated that $P2X_2R$ are necessary for the development of the temporary threshold shift (TTS) evident in auditory brainstem response (ABR) electrophysiological measurements of hearing in wildtype mice exposed to sustained, moderately loud sound; mice null for the *P2rx2* gene (*P2rx2*^{−/−}) that encodes the $P2X_2R$ subunit failed to develop TTS [9]. This study utilised cumulative noise exposure from 10 min to 2 h to determine the time course for development of the TTS (time constant estimated at ~20 min). Without this $P2X_2R$ -mediated hearing adaptation, mice and humans with loss of function *P2RX2* mutations are particularly vulnerable to NIHL and also to age-related hearing loss [9, 15–17]. However, these prior studies did not establish how rapidly purinergic hearing adaptation occurs, which is critical to its ability to protect against loud sounds. Moreover, as adaptation

was demonstrated using ABR, they did not probe the role of outer hair cells in the adaptation process.

The present studies sought improved resolution of the kinetics for the purinergic hearing adaptation and addressed whether the ‘cochlear amplifier’ derived from outer hair cell electromotility contributed to the $P2X_2R$ -dependent reduction in neural output from the cochlea in the face of sustained noise exposure. To achieve this, rates of adaptation of hearing sensitivity with noise exposure were compared between wildtype mice and *P2rx2*^{−/−} mice using ABR and outer hair cell-derived cubic distortion product otoacoustic emission (DPOAE) measurements. The cochlear amplifier provides 40–60 dB gain of the sound energy driving transduction by the inner hair cells, that is coded at the type I spiral ganglion synapses [18–21]. This outer hair cell electromechanical transduction can be directly measured by recording the evoked otoacoustic emissions that are generated by the interaction of sound-evoked cochlear amplifier micromechanics, derived from outer hair cell electromotility, with cochlear basilar membrane mechanics summing and reflecting vibrational energy back through the ossicular chain to the tympanic membrane and hence generating detectable sound in the ear canal [22]. Using two sound drivers enables detection via a microphone pickup in the ear canal, where the cubic ($2f_1-f_2$) DPOAE is prominent and reports the gain of the cochlear amplifier [23, 24]. The data show that the rate of purinergic hearing adaptation is considerably faster than our previous estimate and stems from a reduction of cochlear amplifier gain.

Materials and methods

Animals

Male and female (9–17 weeks) wildtype (*P2rx2*^{+/+}) and *P2rx2*-null (*P2rx2*^{−/−}) mice on a C57Bl/6J background (Australian BioResources, Moss Vale, NSW, Australia) were used for this study. Following previous optimisation of anaesthesia for hearing testing [25], for ABR hearing testing, mice were anaesthetised using intraperitoneal injections (i.p.) of a ketamine (40 mg/kg)/xylazine (8 mg/kg)/acepromazine (0.5 mg/kg) (k/x/a) cocktail; for DPOAE measurements, isoflurane anaesthesia was used (4% induction, 1–1.5% maintenance with O_2). The level of anaesthesia (indicated by breath rate and heart rate) and the O_2 saturation of the mice were monitored using a pulse oximetry system (MouseOx, STARR Life Sciences). The depth of anaesthesia was further assessed by lack of tail-pinch response. Dehydration was prevented by hydrating with 0.9% saline i.p. during the experiments (approximately every 30 min). The core body temperature of the mice was clamped at 37 °C by feedback control using a heating pad (Right Temp, Able Scientific). To prevent

Fig. 1 ATP-gated ion channel $P2X_2$ subunit ($P2X_2R$) distribution in the adult C57Bl/6J mouse organ of Corti. The $P2X_2R$ is detected by confocal immunofluorescence using a rabbit anti-rat $P2X_2R$ primary IgG antibody, labelled via an anti-rabbit Alexa 594 secondary antibody to yield a red signal. The image is a 50μm cryosection through the organ of Corti, showing prominent signal for the $P2X_2R$ protein in the inner (IHC) and outer (OHC) hair cells, and pillar cells (PC). The labelling of these cells is prominent on the apical surface facing the endolymphatic compartment (forming part of the reticular lamina), including in the hair cells, the cuticular plate region and on the stereocilia (*). DAPI staining of the cell nuclei was imaged using multiphoton microscopy; unpublished from [9]. DC, Deiters' cells; HC, Hensen cells

corneal drying, ophthalmic ointment was applied to the eyes once the animal was anaesthetised. After testing, mice were allowed to recover in their home cage on a 37 °C heat pad (Gaymar Industries, Inc., NY, USA) and monitored hourly until full recovery (~30 min for mice under isoflurane anaesthetic and ~4 h for mice under the k/x/a anaesthesia). At the completion of the studies, the animals were euthanised using pentobarbital (Virbac (Australia), 100 mg/kg of body weight at 100 mg/ml).

Hearing function tests

Hearing testing was carried out in a sound-attenuating chamber (Sonora Technology, Japan) using an auditory-evoked potential ABR and DPOAE workstation (TDT system 3 with RX6 and RX6-2 signal processors, Tucker Davis Technologies (TDT), Ft Lauderdale, FL, USA) with BioSig32 software. Sound levels were calibrated using a one-quarter-inch free field calibration microphone (model 7016; ACO Co., Ltd., Japan).

Electrostatic speakers (EC-1, TDT) controlled by the TDT system using BioSigRP software were used to evoke ABR potentials (16 kHz 5 ms tone pips; 0.5 ms rise/fall time, delivered at 10/s) and to generate two primary tones of equal intensity (f_1 and f_2 ; f_2/f_1 ratio: 1.25 about 16 kHz) for the mouse DPOAE recordings.

For ABR recordings, sub-dermal platinum needle electrodes were inserted at the vertex (+), over the mastoid process (−), and with a ground electrode in the hind flank [25]. ABR potentials were amplified, filtered and averaged 512 times, with sound pressure levels (SPL) decreasing in 2.5 dB steps from 70 dB SPL to 10 dB SPL below threshold. The threshold level was defined as the intensity level where an ABR waveform above the noise floor (100 nV) could still be visually detected.

The cubic ($2f_1-f_2$) DPOAEs [25] were detected using a microphone (ER-B10+, Etymotic Research, IL, USA) coupled to the ear canal, driven by the paired primary tones (168 ms duration, 6/s from 0 to 70 dB in 2.5 dB SPL steps), with 50 sound stimuli analysed by Fast Fourier transformation. The threshold level for DPOAEs was defined as the sound level producing a cubic DPOAE with an amplitude of 5 dB above the noise floor. The noise floor was determined as an average of two points on either side of the cubic DPOAE.

Noise exposure studies

There were four studies. These utilised broadband white noise (8–32 kHz for Study 1; 9–32 kHz for Studies 2–4) with an intensity of 85 dB SPL generated using custom software to create a bandpass-delineated (symmetrical Bessel 2nd order roll-off) white noise signal using a D-A interface (NI PCI-6221, National Instruments, USA) driving a MF1 speaker

via an SA1 amplifier (TDT System 3, TDT), coupled to the external ear canal for unilateral close-field exposure.

Hearing function tests (ABR and DPOAE) were measured before noise exposure (t_0), and at a series of time points after cumulative noise exposure in four studies: Study 1 (ABR): 7.5 min ($t_{7.5}$), 17.5 min ($t_{17.5}$) and 37.5 min ($t_{37.5}$) (k/x/a anaesthesia); Study 2 (DPOAE): $t_{7.5}$ and $t_{17.5}$ (isoflurane anaesthesia); Study 3 (ABR): t_5 and t_{10} (k/x/a anaesthesia); Study 4 (DPOAE) $t_{2.5}$, t_5 and $t_{7.5}$ (isoflurane anaesthesia). The opposite ear of the same mouse receiving noise treatment was used for no-noise (unexposed) controls for ABR and DPOAE measurements (Studies 1 and 2), at least 3 days prior to, or after, the noise exposure experiment.

Immunohistochemistry

Mouse cochleae were fixed in 4% paraformaldehyde in phosphate buffer, and then decalcified as described in [9]. Cryosections (50 μ m) were then processed for immunofluorescence free-floating. The tissue was briefly permeabilized using TritonX-100 (0.1% in phosphate-buffered saline (PBS) with 10% normal goat serum; Vector Laboratories, CA, USA) for 2 h at room temperature. The sections were then incubated overnight at 4 °C with the primary antibody (rabbit anti-rat $P2X_2$ receptor; Alomone, 1:1000), followed by washes with PBS, then incubation in secondary antibody (goat anti-rabbit IgG-Alexa 594 secondary antibody; 1:750, Molecular Probes). Nuclear labelling was achieved by incubating the sections in DAPI (4',6-diamidino-2-phenylindole; 1:5000 in PBS; Sigma) for 5 min prior to the final washes. The sections were then washed in PBS and mounted (Vectashield; Vector Labs). DAPI was imaged using multiphoton imaging (792 nm excitation; 431–488 nm emission). Immunofluorescence was imaged using a Zeiss 710 NLO confocal microscope with 561 nm excitation, 585 nm high-pass emission. The specificity of this antibody was validated in control experiments undertaken using $P2rx2^{(-/-)}$ mouse cochlear tissue in the prior study, data shown here were obtained from [9].

Data analysis

Data are presented as the population mean $\pm$ S.E.M. Statistical analysis was performed using two-way repeated measures (RM) analysis of variance (ANOVA), two-way ANOVA, one-way ANOVA and one-sample t test as indicated in Tables 1, 2 and 3 and figure legends (Sigmaplot®, Systat Software Inc.). Data were tested for normal distribution and Holm-Sidak post hoc analysis was utilised for multiple pairwise comparisons within ANOVA. Time constants for development of ABR threshold shifts were derived from best fit single exponential functions of the form $f = a(1 - e^{-bx})$. The time constant for reduction in the cubic DPOAE amplitude in the $P2rx2^{(+/-)}$ was fitted to the decay function $f = -a +$

Table 1 Statistical summary of ABR threshold shifts with cumulative noise exposure. Silent, refers to the no-noise treatment controls (opposite ear to noise treatment). $P2rx2^{(+/+)}$ noise, $n = 11$ – 12 ; $P2rx2^{(+/+)}$ no-noise, $n = 11$; $P2rx2^{(-/-)}$ noise, $n = 9$ – 10 ; $P2rx2^{(-/-)}$ no-noise, $n = 9$; n.s. = not significant; two-way RM ANOVA with Holm-Sidak post hoc multiple pairwise comparisons. t7.5 = 7.5 min, t17.5 = 17.5 min, t37.5 = 37.5 min

16 kHz ABR threshold shifts

Treatment Genotype time point	Silent vs. noise $P2rx2^{(+/+)}$	Silent vs. noise $P2rx2^{(-/-)}$	Silent $P2rx2^{(+/+)}$ vs. $P2rx2^{(-/-)}$	Noise $P2rx2^{(+/+)}$ vs. $P2rx2^{(-/-)}$	Time point	Noise $P2rx2^{(+/+)}$	Noise $P2rx2^{(-/-)}$
t7.5	< 0.001	0.999 n.s.	0.432 n.s.	< 0.001	t7.5 vs. t17.5	0.366 n.s.	0.12 n.s.
t17.5	< 0.001	0.09 n.s.	0.701 n.s.	0.004	t17.5 vs. t37.5	0.783 n.s.	0.993 n.s.
t37.5	< 0.001	0.992 n.s.	0.07 n.s.	0.002			

$a(e^{-bx})$; $P2rx2^{(-/-)}$ DPOAE decay was fitted to a linear regression function of the form $f = -a - bx$.

Results

Study 1: purinergic hearing adaptation measured by ABR across 7.5–37.5-min noise

Study 1 validated the ABR TTS profile with noise exposure in $P2rx2^{(+/+)}$ mice and confirmed the absence of this purinergic hearing adaptation in $P2rx2^{(-/-)}$ mice [9] alongside unexposed controls for both genotypes (littermates) to assure stability of hearing function during the k/x/a anaesthesia. ABR measurements were obtained using 16kHz tone pips undertaken before and during exposure to 85 dB SPL cumulative noise of 7.5 min (t7.5), 17.5 min (t17.5) and 37.5 min (t37.5) for $P2rx2^{(+/+)}$ and $P2rx2^{(-/-)}$ mice. The silent control groups involved re-testing of the opposite (unexposed) ears of the mice on a different day. Figure 2a shows examples of ABR recordings at baseline (i.e. before noise exposure), and after a cumulative noise exposure of 17.5 min, where an increase in threshold is apparent for the $P2rx2^{(+/+)}$ mouse but not the $P2rx2^{(-/-)}$ mouse, as previously established [9]. The threshold shift was fully reversible as established by re-measurement 24–96 h post-noise as shown in Online Resource 1. The data from the noise exposure of the $P2rx2^{(+/+)}$ and $P2rx2^{(-/-)}$ groups is

shown in Fig. 2b, where there was significant TTS in the $P2rx2^{(+/+)}$ mice by 7.5 min ($P2rx2^{(+/+)}$ vs. $P2rx2^{(-/-)}$: 5.2 ± 0.6 dB vs. 1.25 ± 1.1 dB respectively; $p < 0.001$; $n = 12$ and $n = 10$; two-way RM ANOVA). This was maintained at 17.5 min cumulative noise ($P2rx2^{(+/+)}$ vs. $P2rx2^{(-/-)}$: 7.9 ± 1.1 dB vs. 3.8 ± 0.9 dB respectively; $p = 0.004$; $n = 12$ and $n = 10$; two-way RM ANOVA) and at 37.5 min noise ($P2rx2^{(+/+)}$ vs. $P2rx2^{(-/-)}$: 10.0 ± 1.7 dB vs. 4.4 ± 1.5 dB respectively; $p = 0.002$; $n = 11$ and $n = 9$; two-way RM ANOVA). These data indicated that the majority of the TTS (purinergic adaptation) occurred within this initial 7.5 min of noise exposure. The significant differences between noise-exposed ears and the unexposed control ears in these $P2rx2^{(+/+)}$ mice show that the TTS development is due to the noise ($P2rx2^{(+/+)}$, t7.5, t17.5, t37.5, $p < 0.001$ for each time point; $n = 11$ – 12 , $P2rx2^{(+/+)}$ no-noise control, $n = 11$; two-way RM ANOVA; Fig. 2b, Table 1), and not due to non-noise-related reduction in hearing sensitivity, such as prolonged anaesthesia. As evident from these data, the $P2rx2^{(-/-)}$ mice showed no significant ABR threshold shift with noise over the study period ($p = 0.134$, $n = 9$ – 10 ; two-way RM ANOVA). This was further established by comparing the noise-exposed against unexposed ears for these $P2rx2^{(-/-)}$ mice, which showed no significant difference at any time point (t7.5, $p = 0.999$; t17.5, $p = 0.09$; t37.5, $p = 0.992$; $n = 9$ – 10 , $P2rx2^{(-/-)}$, $n = 9$, control; two-way RM ANOVA; Fig. 2b, Table 1); where again, baseline hearing

Table 2 Statistical summary of cubic ($2f_1$ – f_2) DPOAE threshold shifts with cumulative noise exposure. Silent, refers to the no-noise treatment controls (opposite ear to noise treatment). $P2rx2^{(+/+)}$, $n = 8$; $P2rx2^{(-/-)}$, $n = 6$; n.s. = not significant; two-way RM ANOVA with Holm-Sidak post

hoc multiple pairwise comparisons, or one-sample t test (one-tailed) where indicated, testing against no change (0 value). t7.5 = 7.5 min, t17.5 = 17.5 min. f_1 and f_2 about 16 kHz

Cubic ($2f_1 - f_2$) DPOAE threshold shifts

Treatment Genotype time point	Silent vs. noise $P2rx2^{(+/+)}$	Silent vs. noise $P2rx2^{(-/-)}$	Silent $P2rx2^{(+/+)}$ vs. $P2rx2^{(-/-)}$	Noise $P2rx2^{(+/+)}$ vs. $P2rx2^{(-/-)}$	Noise $P2rx2^{(+/+)}$ (one-sample t test)	Noise $P2rx2^{(-/-)}$ (one-sample t test)	Time point	Noise $P2rx2^{(+/+)}$	Noise $P2rx2^{(-/-)}$
t7.5	< 0.001	0.485 n.s.	0.955 n.s.	0.002	≤ 0.001	0.012	t7.5 vs. t17.5	0.058 n.s.	0.587 n.s.
t17.5	< 0.001	0.165 n.s.	0.778 n.s.	< 0.001	0.008	0.019	–	–	–

16 kHz cubic ($2f_1 - f_2$) DPOAE amplitudes with an f_1 and f_2 input level of 40 dB SPL *						
Treatment	Genotype	Time point	Noise $P_{2rx}2^{(+/+)}$	$P_{2rx}2^{(-/-)}$	Noise $P_{2rx}2^{(+/+)} \rightarrow P_{2rx}2^{(-/-)}$ (Fig. 4a example; dB)	$P_{2rx}2^{(-/-)}$ example; dB)
t0 vs. t7.5	<0.001	t0	0.907 n.s.	0.341 n.s.	29.61	24.83
t0 vs. t17.5	<0.001	t7.5	0.004	0.044	18.83	23.48
t7.5 vs. t17.5	0.537 n.s.	t17.5	0.05	0.208 n.s.	14.92	21.45
16 kHz cubic ($2f_1 - f_2$) DPOAE growth function[#]						
Genotype	$P_{2rx}2^{(+/+)}$	Genotype	Time point	Noise $P_{2rx}2^{(-/-)}$	Noise $P_{2rx}2^{(+/+)} \rightarrow P_{2rx}2^{(-/-)}$ vs. $P_{2rx}2^{(-/-)}$	
t0 vs. t7.5	<0.001	-	t0	0.927 n.s.	0.909 n.s.	
t0 vs. t17.5	<0.001	-	t7.5	0.024	<0.001	
t7.5 vs. t17.5	0.859 n.s.	0.016	t17.5	0.121 n.s.	0.004	

Overall change in growth function across the time census points between the $P2rx2^{(+/+)}$ and $P2rx2^{(-/-)}$ cohorts was also analysed and showed a significant difference ($p < 0.001$; $n = 8$

Fig. 2 ABR threshold shifts for *P2rx2*^(+/+) mice (wildtype, WT) and *P2rx2*^(-/-) mice (knockout, KO) before and after exposure to moderate noise (85 dB SPL, 8–32 kHz). **a** Examples of ABR traces before (baseline) and after 17.5 min cumulative noise exposure (post-noise) using 2.5 dB steps in level around threshold. The hearing threshold in the *P2rx2*^(+/+) mouse increased from 0 dB SPL to 10 dB SPL after the noise (blue dashed lines). The *P2rx2*^(-/-) mouse ABR threshold was unchanged by the noise exposure (5 dB SPL, red dashed lines). **b** Boxplots with data overlay (open circles) of the ABR threshold shifts after 7.5 min, 17.5 min and 37.5 min of cumulative noise exposure (blue fill *P2rx2*^(+/+); red fill with dots *P2rx2*^(-/-), or no-noise (silent) controls (blue right diagonal stripes, *P2rx2*^(+/+); red left diagonal stripes, *P2rx2*^(-/-); opposite ears tested on a different day). *P2rx2*^(+/+) noise, *n* = 11–12; *P2rx2*^(+/+) no-noise, *n* = 11; *P2rx2*^(-/-) noise, *n* = 9–10; *P2rx2*^(-/-) no-noise, *n* = 9. The noise-induced ABR threshold shift was only significant in the *P2rx2*^(+/+) mice and was largely in place by the 7.5-min after exposure. ABR was assessed using a 16kHz tone pip stimulus. Dashed lines show the mean; solid lines show the median. Error bars represent the 95th percentile; n.s., *p* > 0.05; two-way RM ANOVA; see Table 1 for a statistical summary

Fig. 3 Cubic ($2f_1-f_2$) DPOAE threshold shifts assessed for $P2rx2^{+/+}$ mice (wildtype, WT) and $P2rx2^{-/-}$ mice (knockout, KO) before and after exposure to moderate noise (85 dB SPL, 9–32 kHz). Boxplots with data overlay (open circles) of the cubic DPOAE threshold shifts after 7.5 min and 17.5 min cumulative noise exposure (blue fill $P2rx2^{+/+}$; red fill with dots $P2rx2^{-/-}$), or no-noise (unexposed) controls (blue right diagonal stripes, $P2rx2^{+/+}$; red left diagonal stripes, $P2rx2^{-/-}$; opposite ears tested on a different day) ($P2rx2^{+/+}$, $n = 8$; $P2rx2^{-/-}$, $n = 6$). The noise-induced DPOAE threshold shift was only significant in the $P2rx2^{+/+}$ mice and as for the ABR study, was largely complete by 7.5 min. Dashed lines show the mean; solid lines show the median. Error bars represent the 95th percentile; n.s., $p > 0.05$; two-way RM ANOVA; see Table 2 for a statistical summary. f_1 and f_2 about 16 kHz

for each genotype; f_1 and f_2 input levels of 35–55 dB SPL; Two-way ANOVA on Ranks; Fig. 4b and c, Table 3). While the growth function for the $P2rx2^{+/+}$ mice is maximally shifted to the right after 7.5 min of noise (t0 vs. t7.5, $p < 0.001$; t7.5 vs. t17.5, $p = 0.859$; $n = 8$; Two-way ANOVA on Ranks; Fig. 4b), this did not hold for the $P2rx2^{-/-}$ mice (t0 vs. t7.5, $p = 0.927$; t7.5 vs. t17.5, $p = 0.121$; $n = 8$; Two-way ANOVA on Ranks; Fig. 4c). Furthermore, although the growth function of the $P2rx2^{-/-}$ mice was significantly different after 17.5 min of noise to that prior to noise exposure (t0 vs. t17.5, $p = 0.024$; $n = 8$; two-way ANOVA on Ranks; 35 dB SPL–55 dB SPL input level), the level of rightward shift is significantly smaller than that of the $P2rx2^{+/+}$ mice ($P2rx2^{+/+}$ t7.5 vs. $P2rx2^{-/-}$ t17.5, $p = 0.016$; $P2rx2^{+/+}$ t17.5 vs. $P2rx2^{-/-}$ t17.5, $p = 0.004$; $n = 8$; two-way ANOVA on Ranks).

These data show a direct correlation between $P2X_2$ R-mediated adaptation of hearing sensitivity measured by ABR threshold shift and cubic DPOAE threshold shift, both of which are largely complete within 7.5 min of noise exposure, and that this adaptation is evident as a right-shift in the input-output function of the $P2rx2^{+/+}$ DPOAE. These data indicate that the adaptation is primarily derived from a reduction in sensitivity of electromechanical transduction at the outer hair cell-based cochlear amplifier, rather than at the primary auditory synapses at the level of the inner hair cells.

Study 3: fine temporal resolution of $P2X_2$ R-mediated hearing adaptation from ABR

Studies 1 and 2 confirmed that the $P2X_2$ R-mediated hearing adaptation was largely complete by 7.5 min of noise exposure. In Study 3, we undertook a finer temporal examination of the onset of the adaptation. The mean ABR thresholds for the $P2rx2^{+/+}$ mice increased from 3.8 ± 1.4 dB SPL to 9.6 ± 2.0 dB SPL after 5-min noise ($p = 0.001$, two-way RM ANOVA, $n = 6$). After 10-min noise, the mean ABR threshold was 14.6 ± 1.9 dB SPL (t5 vs. t10, $p = 0.015$). Figure 5 shows ABR threshold shifts (subtracting the baseline threshold from either the t5 or t10 thresholds for each mouse), with ~50% of the TTS occurring by t5. The $P2rx2^{-/-}$ mice ($n = 6$) had equivalent baseline thresholds (5.4 ± 3.1 dB SPL; $p = 0.903$ vs. t0 for $P2rx2^{+/+}$ mice; two-way RM ANOVA). The $P2rx2^{-/-}$ mouse thresholds were 7.1 ± 2.6 dB SPL at t5; 9.2 ± 2.7 at t10, with no significant change in threshold shifts between t5 and t10 ($p = 0.055$; Fig. 5).

Study 4: fine temporal resolution of $P2X_2$ R-mediated hearing adaptation from DPOAE

As the cubic ($2f_1-f_2$) DPOAE recordings required less signal averaging than the ABR recordings, it was practical to measure the $P2X_2$ R-mediated hearing adaptation at 2.5-min noise exposure separations. This analysis used the same mice as for the Study 3 ABR experiments. The DPOAEs were elicited using 60 dB SPL f_1 and f_2 drivers. The $P2rx2^{+/+}$ mice and $P2rx2^{-/-}$ mice had comparable initial amplitudes (t0) of 25.9 ± 2.3 dB and 28.2 ± 1.9 dB respectively ($p = 0.66$; unpaired t test). The $P2rx2^{+/+}$ and $P2rx2^{-/-}$ mouse DPOAEs diverged with noise (20.6 ± 2.6 dB vs. 26.3 ± 2.2 dB (t2.5), 18.3 ± 2.6 dB vs. 24.2 ± 2.1 dB (t5), 17.3 ± 2.8 dB vs. 23.5 ± 2.0 dB (t7.5)). The overall genotype effect ($P2rx2^{+/+}$ vs. $P2rx2^{-/-}$) across the three noise exposure census points for this study was significant ($p = 0.013$, two-way RM ANOVA, Fig. 6), with the difference in reduction significant from the initial 2.5 min time point (5.3 ± 0.9 dB vs. 1.9 ± 0.6 dB for t2.5, $P2rx2^{+/+}$ vs. $P2rx2^{-/-}$; $p = 0.019$, two-way RM ANOVA). The noise-induced adaptation of the DPOAE output in the $P2rx2^{+/+}$ mice was best fitted by a single exponential decay function, where the derived time constant (τ) = 2.96 min, reconciles well with the rate of adaptation evident in Studies 1 to 3. In contrast, the $P2rx2^{-/-}$ mice exhibited a significantly smaller linear drift in DPOAE over time.

Modelling of ABR adaptation kinetics

For comparison, Fig. 7 provides a synthesis of the ABR threshold shift data from Studies 1 and 3, showing a best fit single exponential function with a time constant (τ) of

Fig. 4 Cubic ($2f_1 - f_2$) DPOAE input/output functions assessed for *P2rx2*^(+/+) mice (wildtype, WT) and *P2rx2*^(-/-) mice (knockout, KO) with exposure to moderate noise (85 dB SPL, 9–32 kHz). **a** Examples of fast Fourier transform traces showing the cubic DPOAE amplitude (DP), at f_1 and f_2 input levels of 40 dB SPL, where noise-induced purinergic adaptation is evident for the *P2rx2*^(+/+) mouse (top) as successive decreases from the starting baseline (29.61 dB, blue dotted line) following 7.5 min (reduced to 18.83 dB) and 17.5 min of cumulative noise exposure (reduced to 14.92 dB). The stability of the cubic DPOAE amplitude during noise exposure in the *P2rx2*^(-/-) mouse indicates the absence of purinergic

adaptation (24.83 dB at baseline (red dotted line), 23.48 dB at 7.5 min noise, and 21.45 dB at 17.5 min of noise). The two primaries (sound stimuli) are indicated as f_1 and f_2 . **b** The noise-induced adaptation of the *P2rx2*^(+/+) (wildtype, WT) cubic DPOAE growth function was highly significant (analysed between input levels of 35–55 dB SPL; two-way ANOVA on Ranks) and largely complete by the 7.5 min census point. **c** There was no significant shift in the cubic DPOAE input/output function in the *P2rx2*^(-/-) ears (n.s., $p > 0.05$). *P2rx2*^(+/+) $n = 8$; *P2rx2*^(-/-) $n = 6$. See Table 3 for a statistical summary. Data in **b** and **c** show mean $\pm$ S.E.M.

5.5 min for the *P2rx2*^(+/+) mice and $\tau = 11.3$ min for the *P2rx2*^(-/-) mice ($p < 0.001$, two-way ANOVA on Ranks). The *P2rx2*-specific ABR development was derived from subtracting the *P2rx2*^(-/-) data from that of the *P2rx2*^(+/+) (Δ ; Fig. 7 inset), with the best fit single exponential

function; $\tau = 3.46$ min. This closely matches the noise-induced adaptation of the cubic DPOAE ($\tau = 2.96$ min) and reinforces the evidence that the cochlear amplifier is the primary driver of the P2X₂R-dependent adaptation to noise.

Fig. 5 Development of the ABR threshold shifts during noise exposure. *P2rx2*^(+/+) mice (wildtype, WT; blue fill) exhibited approximately half the purinergic hearing adaptation within the first 5 min of noise (85 dB SPL, 9–32 kHz). In contrast, the ABR thresholds of the *P2rx2*^(-/-) mice (knockout, KO; red fill with dots) were stable. ABR was assessed using a 16 kHz tone pip stimulus. $n = 6$ for each genotype; n.s., $p > 0.05$; two-way RM ANOVA). Dashed lines show the mean; solid lines show the median. Error bars represent the 95th percentile

Discussion

These four mouse studies resolved the onset kinetics of P2X₂R-dependent hearing adaptation to noise exposure, finding that the time constant was ~ 3 min, whether measured by ABR threshold shift, or reduction in cubic DPOAE amplitude. This is much faster than our previous analysis of P2X₂R-dependent adaptation of ABR thresholds ($\tau \sim 20$ min; Fig. 2a, [9]) which was based on ABR threshold measurements commencing after 10 min of noise and extending to 2 h of cumulative noise exposure. We show here that the noise-induced increase in the ABR thresholds in the wildtype mice is largely complete by 7.5 min, whereas minor non-*P2rx2*-dependent drift in thresholds (evident in the *P2rx2*^(-/-) mice) had slower kinetics that evidently confounded the original estimate. The finding that the adaptation rate of the cubic DPOAE matches the adaptation of the ABR indicates that this reversible noise-induced loss of hearing sensitivity lies with the cochlear amplifier. Evidently, noise-induced release of ATP from the cochlear partition tissues activates organ of Corti P2X₂R-type ATP-gated ion channels (Fig. 1), which in turn produces a temporary reduction in the cochlear amplifier gain. While P2X₂R are also expressed by the spiral ganglion neurons [13, 14, 26], the data do not support a potential neuromodulatory action on auditory neurotransmission as a contributor to the noise-induced ABR threshold shift.

While the present studies make it clear that changes in the cochlear amplifier are responsible for purinergic hearing adaptation to noise, the actual mechanism has yet to be determined.

Fig. 6 Development of the cubic ($2f_1 - f_2$) DPOAE adaptation during noise exposure. *P2rx2*^(+/+) mice (wildtype, WT; blue squares) exposed to noise (85 dB SPL, 9–32 kHz) exhibited an exponential decline in the amplitude of the DPOAE (f_1 and f_2 at 60 dB SPL, about 16 kHz) best fitted by the function $f = -9.30 + 9.30e^{-0.336x}$ (black solid line, $R^2 = 1.0$), corresponding to a time constant (τ) = 2.96 min. In contrast, the *P2rx2*^(-/-) mice (knockout, KO; red circles) showed a small linear drift in the cubic DPOAE output during the 7.5 min cumulative noise exposure (best fitted by the linear regression function $f = -0.237 - 0.654x$, $R^2 = 0.96$, black dashed line). Green bars indicate noise exposure. $n = 6$ for each genotype; p values as shown compare genotypes; two-way RM ANOVA

Injection of ATP into the cochlear scala media chamber in guinea pigs and mice elicits an increase in conductance across the cochlear partition, and this is absent in the *P2rx2*^(-/-) mice; indicating that P2X₂Rs are the only ATP-gated ion channels and the exclusive transepithelial current shunt pathway for purinergic signalling in the cochlea [9, 27]. The outer hair cells must be a major component of this ATP signalling pathway, as they have been found to have high expression levels of ATP-gated ion channels functionally localised to the endolymph-facing apical (sensory) pole [12, 26, 28, 29], and in the *P2rx2*^(-/-) mice, voltage-clamp studies found a complete loss of ATP-activated current, indicating that in the wildtype outer hair cells, the ion channels are only assembled from P2X₂R subunits [9]. The electromotile properties of the outer hair cells underlie dynamic cochlear micromechanics, referred to as the cochlear amplifier, which confers up to ~ 40 dB of hearing sensitivity [19, 20, 30]. It has been suggested that the ATP-gated shunt conductance across the outer hair cells may be a paracrine signalling mechanism controlling cochlear amplifier gain [27]. However, activation of the P2X₂R in the cochlear partition by noise-induced ATP release would be limited to the duration of the noise exposure, due to the rapid hydrolysis of released ATP by extracellular nucleotidases [31, 32]. This is evident from the recovery of the endocochlear potential (EP) within minutes following injection of ATP into the scala media [27]. In contrast, the recovery of the noise-induced P2X₂R-mediated ABR TTS

Fig. 7 Modelling of kinetics of purinergic adaptation from ABR threshold shifts during noise exposure. Best fit single exponential fit to the growth of the ABR threshold shift with cumulative broadband noise exposure (85 dB SPL; derived from Figs. 2b and 5; mean $\pm$ S.E.M.; $n = 6$ –12). $P2rx2^{+/+}$ mice (wildtype, WT; blue squares), $f = 9.66(1 - e^{-0.180x})$ (black solid line; $R^2 = 0.45$), corresponding to a time constant (τ) = 5.5 min; $P2rx2^{-/-}$ mice (knockout, KO; red circles), $f = 4.72(1 - e^{-0.088x})$ (black dashed line; $R^2 = 0.74$), corresponding to $\tau = 11.3$ min ($P2rx2^{+/+}$ vs. $P2rx2^{-/-}$, $p < 0.001$; two-way ANOVA on Ranks on differences between means across time points). Inset, $P2rx2$ -specific development of the ABR ($P2rx2^{-/-}$ data subtracted from $P2rx2^{+/+}$ data; $f = 5.31(1 - e^{-0.289x})$), corresponding to $\tau = 3.46$ min. Green bars indicate noise exposure

has a time constant of 12.3 h [9], which indicates that the sustained suppression of the cochlear amplifier gain involves long-acting secondary mechanisms activated by ATP-gated ion channels. Given that the supporting cells surrounding the outer hair cells also express $P2X_2R$, noise-induced activation of the ATP-gated ion channels in those cells (particularly Deiters' cells) may well contribute to the sustained suppression of the cochlear micromechanics, possibly mediated by Ca^{2+} signalling [33–35].

It is also possible that the EP contributes to the purinergic hearing adaptation; however, the EP is not subject to sustained changes following noise at the levels used here that selectively activate the $P2X_2R$ -specific threshold shifts. The EP is a biopotential within scala media ($\sim +100$ mV) that complements the negative membrane potential of the hair cells to provide the driving force for outer hair cell forward and reverse transduction [36, 37]. Many studies, using guinea pigs or mice, have shown that the much higher noise levels than those used in the present study are required to cause a sustained drop in EP (~ 98 dB SPL; [38–45]). Moreover, Hirose and Liberman [45] showed that despite a temporary threshold shift of 40 dB after 94 dB SPL noise exposure for 2 h, the EP was unaffected in CBA/CaJ mice. Similar results have been observed by Ohlemiller and colleagues [39], where they reported that the EP in C57Bl/6 mice (the strain used in the current study) was unaffected by noise until 110 dB SPL (supported by earlier studies [38, 40]).

Purinergic hearing adaptation, which is a sustained local cochlear paracrine humoral mechanism, complements the more sensitive and dynamic medial olivocochlear efferent system which causes contralateral and ipsilateral suppression of the cochlear amplifier as a negative feedback neural circuit driven by input from the type II spiral ganglion innervation of the outer hair cells [8]. The suppression of the outer hair cells by the medial olivocochlear efferents engages in the sub-second time domain at moderate to loud sound levels (tending to saturate around 85 dB SPL), exhibits adaptation within seconds, is mapped to the exposure sound frequency to modulate auditory nerve firing and confers protection from noise-induced hearing loss [8, 46–48]. In the current study, ipsilateral medial olivocochlear efferent suppression (due to close-field noise presentation to the test ear) may have contributed to the background adaptation of the cubic DPOAE measurements (Fig. 6, $P2rx2^{-/-}$ mice), but this would have been less than 3 dB, based on a study using quadratic ($f_2 - f_1$) DPOAE measurements, which are more sensitive at measuring olivocochlear efferent suppression [8]. Evidently, purinergic adaptation to noise engages broadly towards the upper physiological levels of sustained sound exposure, where basilar membrane mechanics are dominated by the physical properties of its component structures. At these levels, purinergic suppression of the cochlear amplifier effectively extends the dynamic range for encoding sound. The physical stress of sound vibration is likely to be the driver for release of ATP from the tissues lining the cochlear partition, via vesicles and through connexins and pannexin channels [49–53]. With prolonged noise exposure above physiologic levels, purinergic adaptation can protect the organ of Corti from intrinsic overstimulation which leads to glutamate excitotoxicity at the spiral ganglion neurons and wider sensorineural pathophysiology. This is evident from acute and chronic studies of the hearing in $P2rx2^{-/-}$ mice exposed to loud noise. $P2rx2^{-/-}$ mice developed permanent hearing loss after only 30 min of 95 dB SPL 8–16 kHz band noise, while the same exposure has no permanent impact on wildtype controls [9]. Similarly, chronic exposure to environmental level noise (75 dB SPL) up to 17 months of age produced accelerated high-frequency hearing loss, as well as organ of Corti hair cell and supporting cell loss, in $P2rx2^{-/-}$ mice, but not in wild-types [15].

The vulnerability of the cochlea to acoustic overstimulation in the absence of $P2X_2R$ expression can be extended to the human condition. Alongside the evaluation of purinergic adaptation to noise in the $P2rx2^{-/-}$ mouse model, loss of function of the $P2X_2R$ was identified in two independent Chinese families previously characterised as having DNFA41 autosomal dominant progressive sensorineural hearing loss [15], where a c.178G>T (p.Val60Leu) point mutation in the $P2RX2$ gene co-segregated with the DNFA41-based hearing loss. Loss of function was determined by the absence of ATP-gated inward cation current in recombinant HEK293 cells expressing this mutation. The mutation carriers exhibited

progressively worsening hearing loss over 40 years, ending with profound hearing loss (> 50 dB HL), with a record of exposure to environmental noise exposure exacerbating this hearing loss at higher frequencies (> 1 kHz), reminiscent of the findings in homozygous *P2rx2*^(-/-) mice in noisy as compared to quiet environmental chambers [15]. While mutations in the *P2RX2* gene appear to be rare, additional mutations have subsequently been identified through deep sequencing after diagnosis of hearing loss. This includes a missense mutation (p.Gly353Arg) in an Italian family [17], and a c.601G>A (p.Asp201Tyr) mutation detected in two members of a family in the Nagano region of Japan [16].

Within human populations, genetic variance across all the associated aspects of the P2X₂R signalling cascade, including ATP release mechanisms (such as variance in pannexin hemichannels), extracellular ATP hydrolysis (variance in ecto-ATPases), alongside the emerging variance in P2X₂R-type ATP-gated ion channel properties, is likely to contribute to the broad differential vulnerability to noise-induced hearing loss. The expansion of knowledge on functional genomics of these signalling elements in human populations will facilitate further development of transgenic mouse models for probing the physiology around purinergic hearing adaptation.

Conclusion

Our data strongly support the idea that purinergic hearing adaptation to sustained elevation in sound levels occurs upstream of the inner hair cell–spiral ganglion synapses, and that the most likely mechanism involves the reduction in sensitivity of outer hair cell–based cochlear amplifier. They also demonstrate that this adaptation process is fully engaged within a few minutes of noise exposure.

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Compliance with ethical standards

Conflicts of interest Jennie M. E. Cederholm declares that he/she has no conflict of interest.

Allen F. Ryan declares that he/she has no conflict of interest.

Gary D. Housley declares that he/she has no conflict of interest.

Ethical approval All experimental procedures for the mice were reviewed and approved by the UNSW Sydney Animal Care and Ethics Committee.

Declaration of interest statement The authors declare that the findings of this study form part of a patent filing by New South Innovations Pty Ltd., the commercialization arm of UNSW Sydney (the assignee), for a method for predicting vulnerability to hearing loss.

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