

Unmyelinated type II afferent neurons report cochlear damage

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In the mammalian cochlea, acoustic information is carried to the brain by the predominant (95%) large-diameter, myelinated type I afferents, each of which is postsynaptic to a single inner hair cell. The remaining thin, unmyelinated type II afferents extend hundreds of microns along the cochlear duct to contact many outer hair cells. Despite this extensive arbor, type II afferents are weakly activated by outer hair cell transmitter release and are insensitive to sound. Intriguingly, type II afferents remain intact in damaged regions of the cochlea. Here, we show that type II afferents are activated when outer hair cells are damaged. This response depends on both ionotropic (P2X) and metabotropic (P2Y) purinergic receptors, binding ATP released from nearby supporting cells in response to hair cell damage. Selective activation of P2Y receptors increased type II afferent excitability by the closure of KCNQ-type potassium channels, a potential mechanism for the painful hypersensitivity (that we term “noxacusis” to distinguish from hyperacusis without pain) that can accompany hearing loss. Exposure to the KCNQ channel activator retigabine suppressed the type II fiber’s response to hair cell damage. Type II afferents may be the cochlea’s nociceptors, prompting avoidance of further damage to the irreparable inner ear.

type II cochlear afferents | ATP | acoustic trauma | hyperacusis | noxacusis

The mammalian cochlea is the most elaborate of vertebrate auditory organs. The elegantly coiled, mechanically tuned cochlear duct and functional differentiation between inner hair cells (IHCs) and outer hair cells (OHCs), afferent and efferent neuronal connections are among the features that enable the widest acoustic frequency range and most complex vocalizations among vertebrate species. This benefit, however, has been gained at a significant cost in the metabolic and mechanical vulnerability of cochlear hair cells and neurons. Loud sound progressively damages type I afferent neurons and OHCs (1). Once damaged beyond repair, these do not regenerate as they do in nonmammalian vertebrates, suggesting that protective mechanisms should exist to preserve cochlear function. Indeed, middle ear reflexes (2) and efferent inhibition of hair cells (3) can mitigate acoustic trauma to some extent. However, a more effective strategy is simply to avoid or withdraw from sources of trauma, by analogy to limb withdrawal triggered by C-fiber activation in skin.

Here, we provide evidence that unmyelinated type II cochlear afferents can report cochlear trauma, a potential trigger for nociceptive behavior. Hyperactivity of type II neurons could contribute as well to the paradoxical hypersensitivity to loud sound that can accompany hearing loss, despite diminished type I afferent function. Hyperacusis is a comorbidity in 80% of tinnitus patients (4) suggesting common pathogenic mechanisms (5). In the most severe cases, hyperacusis is described as debilitating “ear pain” (6). The response to trauma by type II afferents may relate most directly to such noxious hearing—“noxacusis,” to coin a term. In support of this hypothesis, sparse (~5% of all spiral ganglion neurons) unmyelinated type II afferents can survive cochlear damage (7, 8), are insensitive to sound (9, 10), but are activated by the algogenic ligand adenosine triphosphate (ATP) (11). In contrast to the predominant type I afferents that contact IHCs to encode the information content of sound, type II afferents innervate OHCs,

which are more sensitive to acoustic trauma (12). Here, we show that type II afferents are excited by ATP released from supporting cells around damaged OHCs, revealing cellular mechanisms and potential molecular pathways for inner ear pain.

Results

The Experimental Preparation. To monitor type II afferents, intracellular and extracellular recordings were made from their spiral dendrites under OHCs in the apical turn of the cochlea excised from young rats [postnatal day 7 (P7) to P10] (Fig. 1A). As a proxy for cochlear trauma, individual OHCs were ruptured with a glass microneedle positioned apical to the recording electrode. By rapidly advancing the needle, one to three OHCs were ruptured (Fig. 1B), visualized by the loss of FM1-43 fluorescence specifically taken up through hair cells’ transduction channels (13) (Fig. 1C). The damaged hair cells become round and swollen, and disintegrate within a few minutes after rupture. Equivalent but off-center punctures failed to rupture hair cells and no change was recorded in type II membrane current or potential, ruling out direct mechanical effects of the needle on the type II afferent (Fig. 2B).

ATP Contributes to the Cell Damage-Induced Response. Upon OHC rupture, a large depolarization (23.8 ± 4.5 mV; 14 trials in seven afferents) was recorded from type II afferents (Fig. 2A). In voltage-clamp recordings, a long-lasting inward current was observed (peak current, 111.9 ± 16.4 pA at -70 mV; 10 trials in six fibers) with a 90% decay time of 58.0 ± 9.5 s. A role for ATP in this response was shown by the application of pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) (50 μ M), a P2X receptor antagonist that also partially blocks P2Y4 and P2Y6 receptors. The damage-induced current was greatly abbreviated in PPADS (Fig. 2C)

Significance

Painfully loud sound causes protective or withdrawal responses, rather than continued listening. This differential behavior invites comparison with somatic pain responses driven by the anatomically distinct subset of small-diameter, unmyelinated afferents—C fibers. Like somatic C fibers, unmyelinated type II cochlear afferents differ in size, number, and innervation pattern from type I afferents that encode sound. Here, we show that type II afferents are excited during cochlear tissue damage in part by the algogenic cytoplasmic metabolite adenosine triphosphate (ATP). This finding, together with previous evidence that type II afferents respond weakly to synaptic transmission from cochlear hair cells, and normally are insensitive to sound, supports the identification of type II afferents as cochlear nociceptors, mediating the sensation of painfully loud sound.

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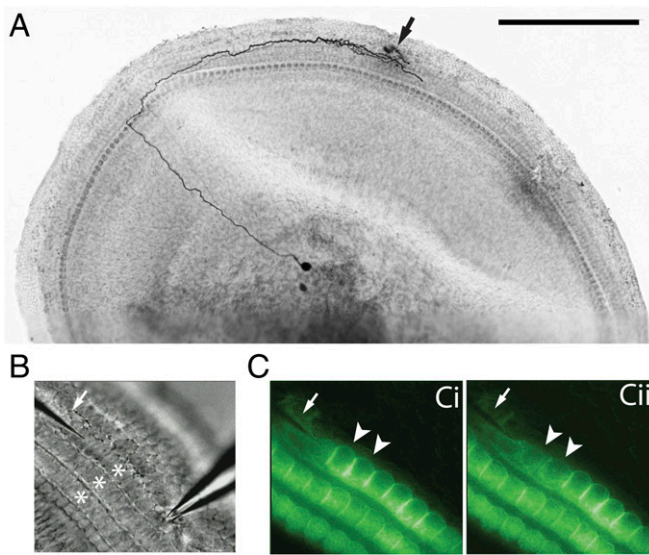


Fig. 1. The experimental preparation. (A) Whole mount of the apical turn of a P8 rat cochlea (apex to the *Left*), type II afferent filled with biocytin. Typical recording site (arrow) and OHC damage site were within the branched synaptic input zone. (Scale bar: 250 μm .) (B) Electrode (*Right*) recording from a type II afferent (out of focus) under OHC rows (stars). The glass needle (arrow) poised to ablate one to three OHCs per trial. (C) Two FM1-43-loaded OHCs [arrowheads (*Ci*)] were ruptured and lost fluorescence (*Cii*).

(90% decay time, 13.3 ± 7.9 s; $P < 0.001$ compared with control), and charge transfer decreased (from 2.5 ± 0.4 nC in control to 0.8 ± 0.2 nC; $P < 0.001$). This prolonged response is similar to the time course of ATP-dependent calcium waves observed in cochlear supporting cells after hair cell rupture. These minutes-long waves trigger regenerative ATP release through connexin hemichannels (14–17). Accordingly, when the connexin hemichannel blocker carbenoxolone (CBX) was applied to block ATP release from supporting cells, the type II afferent response to hair cell damage was significantly shortened, with a 90% decay time of 13.8 ± 2.8 s and charge transfer of 0.8 ± 0.1 nC (Fig. 2D; $P < 0.001$ for both measures compared with control), suggesting supporting cells as a major source of ATP acting on type II afferents. In contrast, the peak current amplitude in PPADS and CBX was not significantly different from that of control responses. This early peak also was not affected by glutamate receptor block (Fig. S1) but may reflect potassium release from ruptured OHCs acting directly on type II afferents. The amplitude of the early transient was reduced when external potassium was elevated before hair cell rupture, and equivalent inward currents were produced by direct application of 150 mM K^+ saline (mimicking hair cell cytoplasm) (Fig. S2). Such immediate rupture of individual hair cells might occur in vivo (7). More commonly, however, acoustic stress progressively damages OHCs, leading to their eventual death, and is known to increase ATP concentration in cochlear fluids in vivo (18). Thus, the purinergic excitation of type II afferents was examined in greater detail.

Purinergic Receptors in Type II Afferents. Inward current induced in type II afferents by direct application of ATP (50 μM) (55.3 ± 17.7 pA at -60 mV; 10 experiments in seven cells) was significantly reduced by the P2X antagonist, PPADS (Fig. 3A; 3.5 ± 3.4 pA; $P < 0.05$, compared with controls; four experiments in four cells). UTP (100 μM), an agonist of P2Y2, P2Y4, and P2Y6 receptors, evoked a small inward current in voltage clamp at -60 mV (8.9 ± 4.7 pA; 12 experiments in eight cells; Fig. 3B). These results suggest the presence of two distinct purinergic responses

mediated by ligand-gated P2X receptors and G-protein-coupled P2Y receptors, respectively.

A voltage-ramp protocol was used to unveil the ionic mechanism underlying ATP- or UTP-evoked current. Ramp current in the absence of ATP or UTP (Fig. 3C and D, black) was subtracted from that in its presence (Fig. 3C and D, red and blue) to obtain the ATP- or UTP-dependent ramp current (Fig. 3C and D, *Inset*). The ATP-dependent current-voltage (I - V) relation had a positive slope at its reversal near 0 mV (Fig. 3E; representative of five experiments in three cells), consistent with the activation of non-selective cation channels (P2X receptors). In contrast, the UTP-evoked ramp current was entirely inward, activating positive to -70 mV (Fig. 3F; representative of six experiments in six cells), suggesting the closure of voltage-dependent potassium channels by P2Y receptors signaling through second-messenger pathways (19).

P2Y Receptors Close KCNQ Channels to Increase Type II Afferent Excitability. The KCNQ channel antagonist XE-991 reversibly eliminated the UTP-dependent ramp current in type II afferents (Fig. 4A; from 92.7 ± 20.8 to 3.8 ± 4.5 pA at $+40$ mV; four experiments in four cells; $P < 0.01$), and the KCNQ channel opener retigabine reliably activated outward currents (Fig. 4B; 37.9 ± 3.0 pA at -60 mV; four experiments in four cells). These effects suggest that P2Y receptor activation depolarized type II afferents through the closure of KCNQ channels. This is consistent with the small effect of UTP at -60 mV (Fig. 3B) where few voltage-dependent KCNQ channels are open. The relative contribution of P2X and P2Y-KCNQ pathways depends on the local ATP concentration. In submicromolar ATP, the higher-affinity P2Y-KCNQ pathway is preferentially activated. In higher concentrations of ATP, the ionotropic P2X receptors predominate (Fig. S3).

The closure of KCNQ channels by P2Y receptors increased type II fiber excitability. In UTP, the current threshold for type II afferent action potentials was reduced to $78.2 \pm 3.5\%$ of the level required in normal conditions (four experiments in four cells;

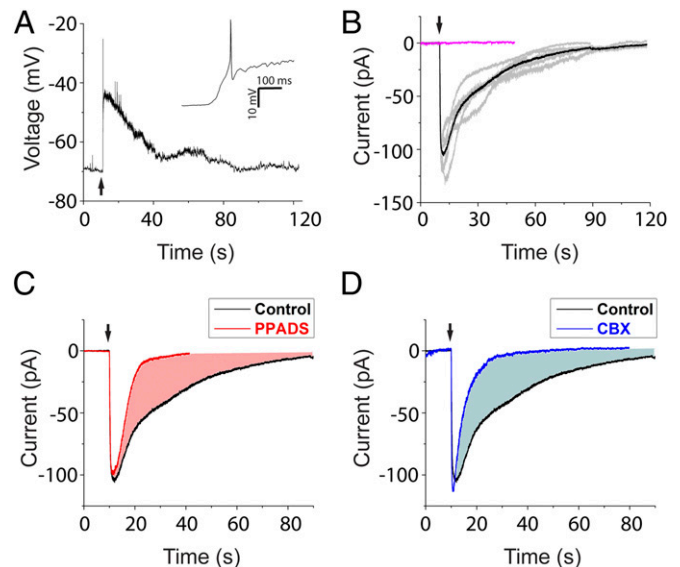


Fig. 2. ATP contributes to cell damage-induced response. (A) OHC ablation depolarized type II afferents and triggered action potentials on the rising phase (*Inset*). (B) Representative traces of damage-induced currents (at -70 mV) from five different type II afferents (gray) and the average (black). Identical needle movement that failed to ablate OHCs induced no current (magenta). (C) Average ablation-induced current in PPADS (red, three trials in three afferents) compared with average control current from B. (D) Average ablation-induced current in CBX (blue, five trials in three afferents) compared with control average.

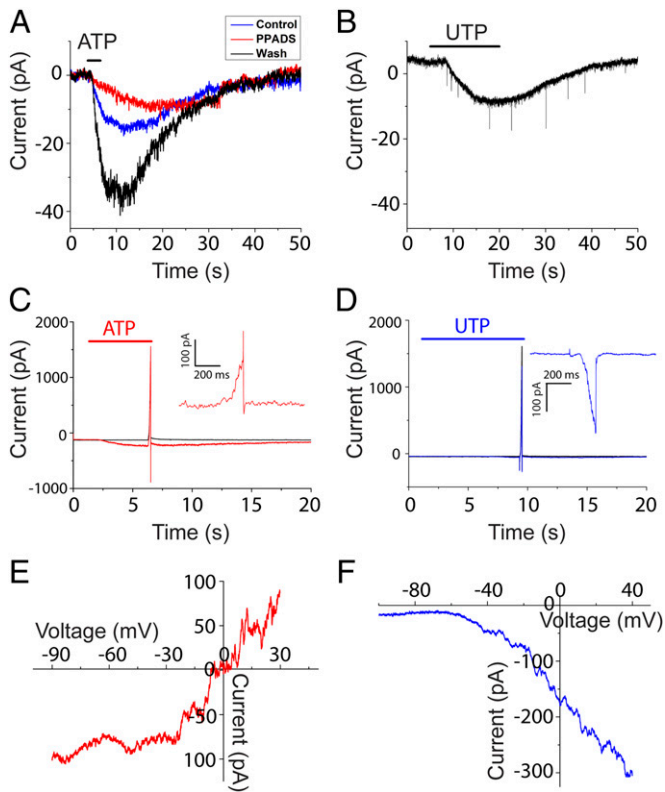


Fig. 3. Purinergic receptors in type II afferents. (A) ATP-evoked inward current could be blocked by the P2X antagonist PPADS (red), with partial recovery (blue). (B) UTP (agonist for P2Y2 and P2Y4 receptor) induced inward current in type II afferents. Excitatory postsynaptic currents were recorded, but their frequency was not significantly increased during UTP application. (C) Overlay of responses from a type II afferent when the holding potential was ramped from -90 to $+30$ mV in normal external solution (black), or after ATP was applied (red). (Inset) Difference current during voltage ramp with and without ATP application. (D) Overlay of responses from a type II afferent when the holding potential was ramped from -110 to $+30$ mV in normal external solution (black), or after UTP was applied (blue). (Inset) Difference current during voltage ramp with and without UTP application. (E and F) I - V relation of ATP and UTP response revealed by voltage-ramp recordings. The current evoked by ATP reversed at 0 mV (red; $n = 3$ afferents) and reversal of UTP-evoked current extrapolated to near -70 mV (blue; $n = 6$ afferents).

$P < 0.01$) (Fig. 4C). Thus, UTP caused a small, but significant increase in excitability, despite the fact that these measurements were made at rest where few KCNQ channels are open (Fig. 3F). The effect of P2Y-mediated KCNQ closure will be greater still when type II afferents are depolarized as during acoustic trauma. Thus, KCNQ channels serve as a promising target to modulate the damage-sensitive type II afferents. Consistent with that proposal, repeated hair cell ablation in the presence of retigabine (KCNQ channel opener) failed to elicit any action potentials in five type II fibers (Fig. 4D), echoing the analgesic effect of retigabine on somatic pain pathways (20).

Discussion

This work provides direct evidence that type II afferents, in addition to sensing glutamate release from OHCs, are activated by cochlear damage in the young rat's cochlea. This observation may help to resolve the decades-long conundrum that type II afferents in vivo are very insensitive to sound (9, 10) and yet presumably carry some information to the auditory brainstem. Likewise, measured ex vivo, synaptic excitation is weak and could activate type II afferents only if all of the presynaptic OHCs were maximally stimulated (21). Alternatively, ATP potently activates type II afferents (11) and

serves as a major contributor to the damage-induced response. ATP can be released into cochlear fluid after tissue stress (even without OHC ablation) in vitro (22) or noise exposure in vivo (18). Experimental ablation of OHCs was shown to initiate ATP-dependent calcium waves in nearby Hensen's cells that further triggers release of ATP through their connexin hemichannels (14–17). P2X2 receptors have been located to the postsynaptic junction in the OHC region in adult guinea pig (23), and P2Y2 receptors have been identified in a small population of spiral ganglion neurons in both adult and neonatal rats (24), suggesting the expression of purinergic receptors in type II neurons. Of interest in this context is the previous report that sensitivity to ATP is reduced in type II afferents after the onset of hearing (11), consistent with the fact that loud sound is not usually painful to normal ears. However, purinergic signaling in the cochlea is up-regulated after noise exposure (25), raising the possibility that type II afferents become more sensitive after damage, in part by increased sensitivity to ATP.

ATP-dependent activation of type II afferents differs from the generation of spontaneous activity in type I afferents by ATP released from cells of Kolliker's organ during cochlear maturation (26). ATP application produced action potentials in type I fibers only via release of glutamate from IHCs, whereas direct effects of ATP on type I afferent membrane current or voltage were small and subthreshold. In contrast, although ATP can evoke glutamate release from OHCs, that source of excitation on its own is insufficient to activate the type II afferent (11, 21).

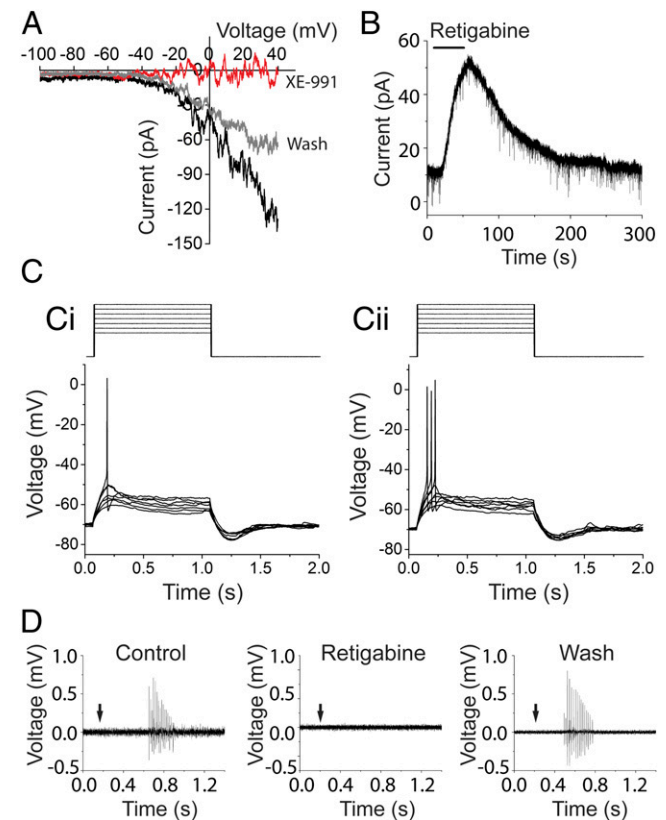


Fig. 4. KCNQ channels regulate type II afferent excitability. (A) XE-991 (KCNQ blocker) reversibly eliminated the UTP-evoked ramp current. I - V relation of UTP before (black), during (red), and after (gray) XE-991 application. (B) Retigabine (KCNQ opener) induced outward current at -60 mV in type II cochlear afferents. (C) Current step protocol (1 pA steps) to evoke action potentials (Ci). In UTP, from the same resting membrane potential, action potentials were evoked by smaller current steps (Cii). (D) The KCNQ channel activator retigabine reversibly prevented the response of type II afferents to OHC ablation ($n = 5$ afferents).

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