

Purinergic signaling in cochlear supporting cells reduces hair cell excitability by increasing the extracellular space

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Abstract Neurons in developing sensory pathways exhibit spontaneous bursts of electrical activity that are critical for survival, maturation and circuit refinement. In the auditory system, intrinsically generated activity arises within the cochlea, but the molecular mechanisms that initiate this activity remain poorly understood. We show that burst firing of mouse inner hair cells prior to hearing onset requires P2RY1 autoreceptors expressed by inner supporting cells. P2RY1 activation triggers K⁺ efflux and depolarization of hair cells, as well as osmotic shrinkage of supporting cells that dramatically increased the extracellular space and speed of K⁺ redistribution. Pharmacological inhibition or genetic disruption of P2RY1 suppressed neuronal burst firing by reducing K⁺ release, but unexpectedly enhanced their tonic firing, as water resorption by supporting cells reduced the extracellular space, slowing K⁺ clearance. These studies indicate that purinergic signaling in supporting cells regulates hair cell excitability by controlling the volume of the extracellular space.

23

Introduction

The developing nervous system must generate, organize, and refine billions of neurons and their connections. While molecular guidance cues forge globally precise neuronal connections between distant brain areas (*Stoeckli, 2018; Dickson, 2002*), the organization of local connections is initially coarse and imprecise (*Dhande et al., 2011; Kirkby et al., 2013; Sretavan and Shatz, 1986*). Coincident with the refinement of topographic maps, nascent circuits experience bursts of intrinsically generated activity that emerge before sensory systems are fully functional (*Kirkby et al., 2013*). This intrinsically generated activity consists of periodic bursts of high frequency firing that promotes the survival and maturation of neurons in sensory pathways (*Blankenship and Feller, 2010; Moody and Bosma, 2005*). The precise patterning of this electrical activity appears crucial for refinement of local connections, as its disruption results in improper formation of topographic maps (*Antón-Bolaños et al., 2019; Burbridge et al., 2014; Xu et al., 2011*) and impaired maturation and specification of sensory neurons (*Shrestha et al., 2018; Sun et al., 2018*). In all sensory systems that have been examined, spontaneous burst firing arises within their respective developing sensory organs, e.g. retina, olfactory bulb, spindle organ, and cochlea (*Blankenship and Feller, 2010*). Although the mechanisms that induce spontaneous activity in the developing retina have been ex-

40 tensively explored, much less is known about the key steps involved in triggering auditory neuron
 41 burst firing in the developing cochlea. Understanding these processes may provide novel insights
 42 into the causes of developmental auditory disorders, such as hypersensitivity to sounds and audi-
 43 tory processing disorders that prevent children from communicating and learning effectively.

44 The mechanisms responsible for initiating spontaneous activity appear to be unique to each
 45 sensory system, reflecting adaptations to the structure and cellular composition of the sensory or-
 46 gans. In the cochlea, two distinct models have been proposed to initiate burst firing of inner hair
 47 cells. One model proposes that burst firing results from intermittent hyperpolarization of tonically
 48 active IHCs by cholinergic efferents (*Johnson et al., 2011; Wang and Bergles, 2014*), which provide
 49 prominent inhibitory input to IHCs prior to hearing onset (*Glowatzki and Fuchs, 2000*). Consistent
 50 with this model, activation of acetylcholine receptors in acutely isolated cochleae caused IHCs to
 51 switch from sustained to burst firing (*Johnson et al., 2011*). However, *in vivo* recordings from audi-
 52 tory brainstem revealed that neuronal burst firing remains, with altered features, in a9 knockout
 53 mice (*Clause et al., 2014*), which lack functional efferent signaling in IHCs (*Johnson et al., 2013*), and
 54 persists in IHCs and auditory neurons in cochleae maintained *in vitro* without functional efferents
 55 (*Johnson et al., 2013*). Thus, cholinergic efferents appear to modulate the temporal characteristics
 56 of bursts, but are not essential to initiate each event.

57 An alternative model proposes that IHCs are induced to fire bursts of action potentials by the
 58 release of K⁺ from nearby inner supporting cells (ISCs), which together form a transient structure
 59 known as Kölliker's organ (Greater Epithelial Ridge) that is prominent prior to hearing onset (*Tritsch
 60 et al., 2010b*). K⁺ release from ISCs occurs following a cascade of events that begins with the spon-
 61 taneous release of ATP and activation of purinergic autoreceptors. Purinergic receptor activation
 62 induces an increase in intracellular Ca²⁺, opening of Ca²⁺-activated Cl⁻ channels (TMEM16A), and
 63 efflux of Cl⁻ and subsequently K⁺ to balance charge (*Tritsch et al., 2007; Wang et al., 2015*). The loss
 64 of ions during each event draws water out of ISCs through osmosis, leading to pronounced shrink-
 65 age (crenation) of ISCs. While these pathways have been extensively studied *in vitro*, the molecular
 66 identity of the purinergic receptors has remained elusive and few manipulations of key steps in
 67 this pathway have been performed *in vivo*, limiting our understanding of how spontaneous activity
 68 is generated at this critical stage of development.

69 Here, we show that the key initial step in generation of spontaneous activity in the auditory
 70 system involves activation of P2RY1 autoreceptors in ISCs. These metabotropic receptors induce
 71 Ca²⁺ release from intracellular stores that allow TMEM16A channels to open. Pharmacological in-
 72 hibition of P2RY1 or genetic deletion of *P2ry1* dramatically reduced burst firing in spiral ganglion
 73 neurons (SGNs) and blocked the coordinated, spatially restricted activation of ISCs, IHCs, and SGNs
 74 in the cochlea. Unexpectedly, P2RY1 activation also promoted the clearance of K⁺ by increasing
 75 the volume of extracellular space, enhancing the diffusion of K⁺ ions away from IHCs. Conversely,
 76 inhibition of P2RY1 reduced the extracellular space and restricted the redistribution of K⁺ within
 77 the cochlear epithelium, causing IHCs to depolarize and fire tonically, demonstrating an important
 78 role for purinergic receptor-mediated extracellular space changes in controlling IHC excitability.
 79 Using *in vivo* widefield epifluorescence imaging of the auditory midbrain in unanesthetized mice,
 80 we show that acute inhibition of P2Y1 receptors dramatically reduced burst firing of auditory neu-
 81 rons in isofrequency domains. Together, these data indicate P2RY1 autoreceptors in non-sensory,
 82 cochlear supporting cells play a crucial role in generating bursts of activity among neurons that will
 83 ultimately process similar frequencies of sound, providing the means to initiate the maturation of
 84 auditory pathways before hearing onset.

85 **Results**

86 **Supporting cell spontaneous currents require calcium release from intracellular
87 stores**

88 Periodic release of ATP from ISCs in the developing cochlea initiates a signaling cascade in these
89 cells that increases intracellular calcium (Ca^{2+}), opens Ca^{2+} -activated Cl^- channels (TMEM16A), and
90 ultimately results in efflux of chloride and K^+ into the extracellular space. Although the increase
91 in intracellular Ca^{2+} following activation of purinergic autoreceptors is sufficient to induce both
92 depolarization and osmotic shrinkage (crenation; *Wang et al. (2015)*), the relative contributions of
93 Ca^{2+} influx (e.g. through Ca^{2+} -permeable, ionotropic P2X receptors) and release from intracellular
94 stores (e.g. following metabotropic P2Y receptor activation) to these cytosolic Ca^{2+} transients is un-
95 clear. To define the signaling pathways engaged by purinergic receptor activation, we examined
96 the sensitivity of spontaneous ISC whole-cell currents and crenations to inhibitors of intracellular
97 Ca^{2+} pathways (*Figure 1A*). Spontaneous inward currents and crenations were abolished following
98 a 15 minute incubation of excised cochlea in BAPTA-AM (100 μM), a cell permeant Ca^{2+} chelator
99 (*Figure 1B-F*), and after depleting intracellular Ca^{2+} stores with thapsigargin (2 μM), an inhibitor of
100 endoplasmic reticulum Ca^{2+} -ATPase (*Figure 1B-F*). These data suggest that Ca^{2+} release from intra-
101 cellular stores is necessary for spontaneous electrical activity in ISCs.

102 Metabotropic G_q-coupled receptors typically induce PLC-mediated cleavage of phosphatidyli-
103 nositol 4,5-bisphosphate (PIP₂) and subsequent binding of inositol trisphosphate (IP₃) to IP₃ receptor-
104 channels on the endoplasmic reticulum to release Ca^{2+} into the cytoplasm. To investigate if PLC
105 signaling is required for generation of spontaneous activity in ISCs, we recorded spontaneous cur-
106 rents and crenations from ISCs in the presence of U73122 (10 μM), a PLC inhibitor, and U73343
107 (10 μM), an inactive succinimide analog. The frequency of spontaneous currents and crenations
108 were dramatically reduced following U73122 incubation, but not U73343 (*Figure 1B-F*); the ampli-
109 tudes and charge transfer of residual activity also trended lower during PLC inhibition, but this did
110 not reach significance due to high variance in the sizes of these responses (*Figure 1B-F*). Together,
111 these results suggest that engagement of a G_q-coupled purinergic autoreceptor is a critical first
112 step in initiating PLC-mediated Ca^{2+} release from intracellular stores and subsequent activation of
113 TMEM16A channels.

114 **The metabotropic purinergic receptor P2Y1 is highly expressed by supporting cells**

115 There are eight members of the metabotropic purinergic receptor family in mouse, four of which
116 are G_q-coupled (P2RY1, P2RY2, P2RY4, and P2RY6). Gene expression studies in the developing
117 mouse cochlea revealed that non-sensory cells express *P2ry1* mRNA at high levels (*Scheffer et al.,*
118 *2015*), >100 fold higher than any other P2ry (*Figure 2A*) and that expression of this receptor progres-
119 sively increases during early postnatal development (*Figure 2A*, inset) concurrent with increases
120 in spontaneous activity (*Tritsch and Bergles, 2010*). To determine which cells in the sensory ep-
121 ithelium express P2RY1, we isolated cochleae from *P2ry1-LacZ* reporter mice and performed X-gal
122 staining. Intense blue labeling was present along the entire length of the cochlea within Kölliker's
123 organ (Greater Epithelial Ridge; *Figure 2B*). Cross-sections of cochlea revealed that staining was
124 present within ISCs, but not IHCs (Myosin VIIA, *Figure 2C*), indicating that P2RY1 is properly local-
125 ized to sense ATP release from ISCs prior to hearing onset.

126 **P2RY1 signaling is required for spontaneous activity in ISCs and IHCs**

127 To determine if P2RY1 is responsible for spontaneous ATP-mediated currents in ISCs, we examined
128 the sensitivity of these responses and associated crenations to the P2RY1 antagonist MRS2500 (*Fig-*
129 *ure 3A,B*). Acute inhibition of P2RY1 with MRS2500 (1 μM) markedly reduced both spontaneous ISC
130 currents (*Figure 3B,C*) and crenations (*Figure 3D,E*); near complete inhibition occurred within min-
131 utes at both room temperature (*Figure 3B,C*) and near physiological temperature (*Figure 3-Figure*
132 *Supplement 1A-G*), with only sporadic, small amplitude events remaining that were not mediated

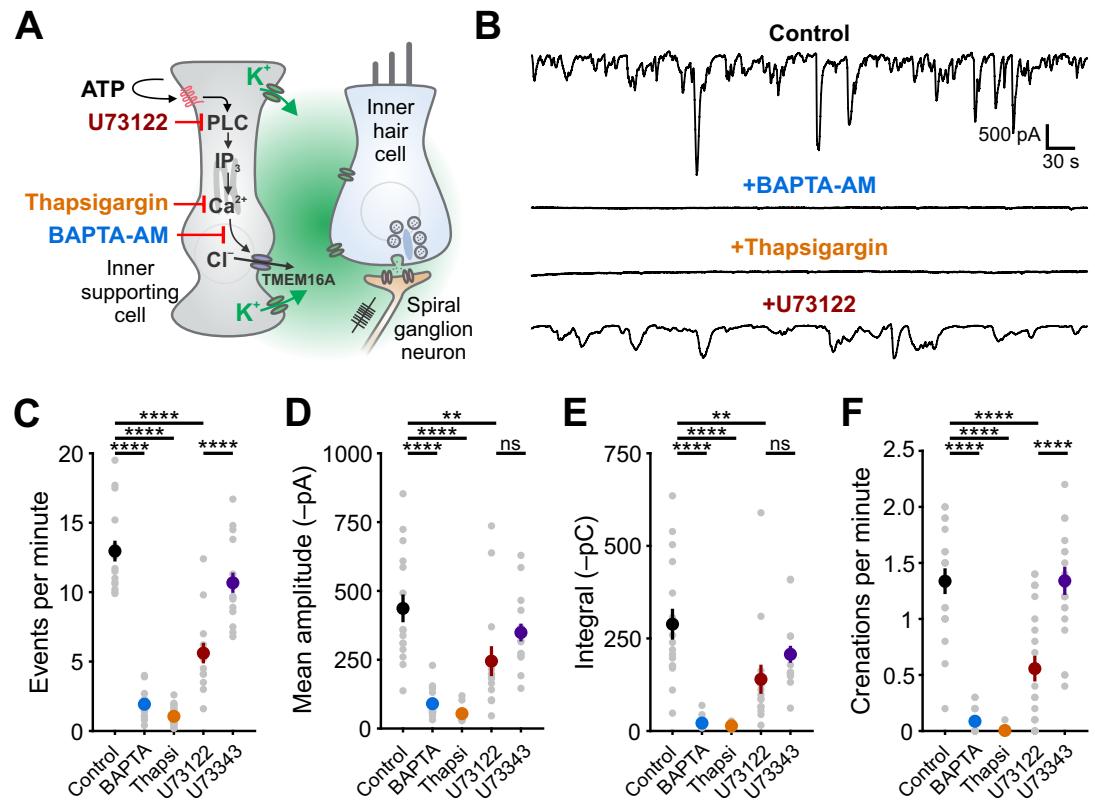


Figure 1. Ca²⁺ release from intracellular stores is required for spontaneous currents and crenation in inner supporting cells. **(A)** Model of ATP-mediated depolarization of inner hair cells. ATP: adenosine triphosphate, PLC: phospholipase C, IP₃: inositol triphosphate, TMEM16A: transmembrane member 16A (Ca²⁺-activated Cl⁻ channel). Inhibitors of key steps in this pathway are indicated. **(B)** Whole-cell voltage-clamp recordings from inner supporting cells after pre-incubating with indicated inhibitors. **(C)** Quantification of ISC spontaneous current frequency in the presence of inhibitors of the G_q pathway. Data shown as mean ± SEM. n = 16 cells (control), 16 cells (BAPTA-AM; 100 μM), 20 cells (Thapsigargin; 2 μM), 14 cells (U73122; 10 μM), and 16 cells (U73343; 10 μM). ****p<5e-5, one-way ANOVA. **(D)** Quantification of ISC spontaneous current amplitude in the presence of inhibitors of the G_q pathway. Data shown as mean ± SEM. n values are reported in (C) (one-way ANOVA; ****p<5e-5, **p<0.005, ns: not significant). **(E)** Quantification of ISC spontaneous current charge transfer (integral) in the presence of inhibitors of the G_q pathway. Data shown as mean ± SEM. n values are reported in (C) (one-way ANOVA; ****p<5e-5, **p<0.005, ns: not significant). **(F)** Quantification of ISC crenation (cell shrinkage) frequency in the presence of inhibitors of the G_q pathway. Data shown as mean ± SEM. n values are reported in (C) (one-way ANOVA; ****p<5e-5, **p<0.005, ns: not significant).

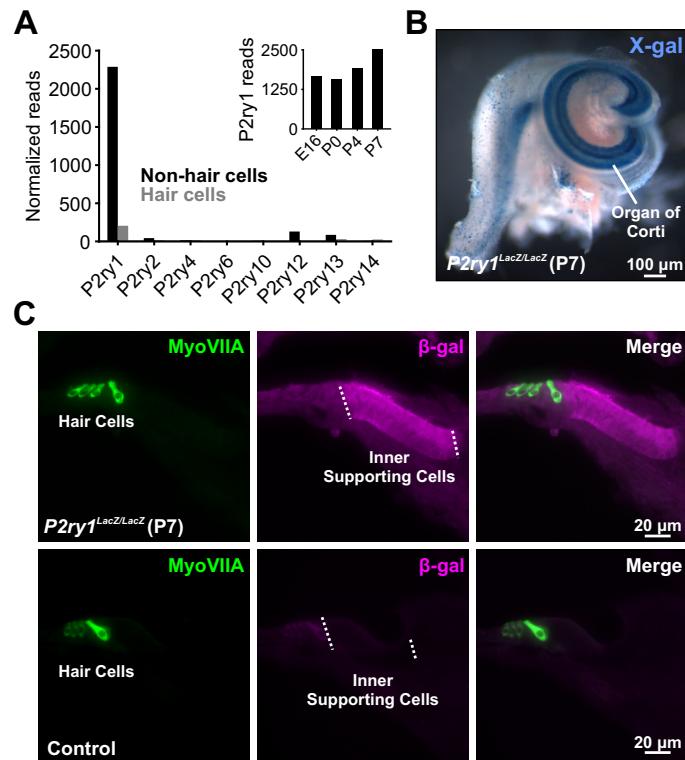


Figure 2. The metabotropic P2Y1 receptor is highly expressed by ISCs. **(A)** Expression levels of metabotropic purinergic receptors in hair cells (grey) and non-sensory cells (black) of the developing cochlea (postnatal day 7, P7). (inset) *P2ry1* expression in non-sensory cells over development. Data adapted from Scheffer et al. (2015). **(B)** Image of a cochlea following X-gal reaction in *P2ry1-LacZ* reporter mice. **(C)** Immunostaining for β -galactosidase in cochleae from P7 *P2ry1-LacZ* (top) and control (bottom) cochlea.

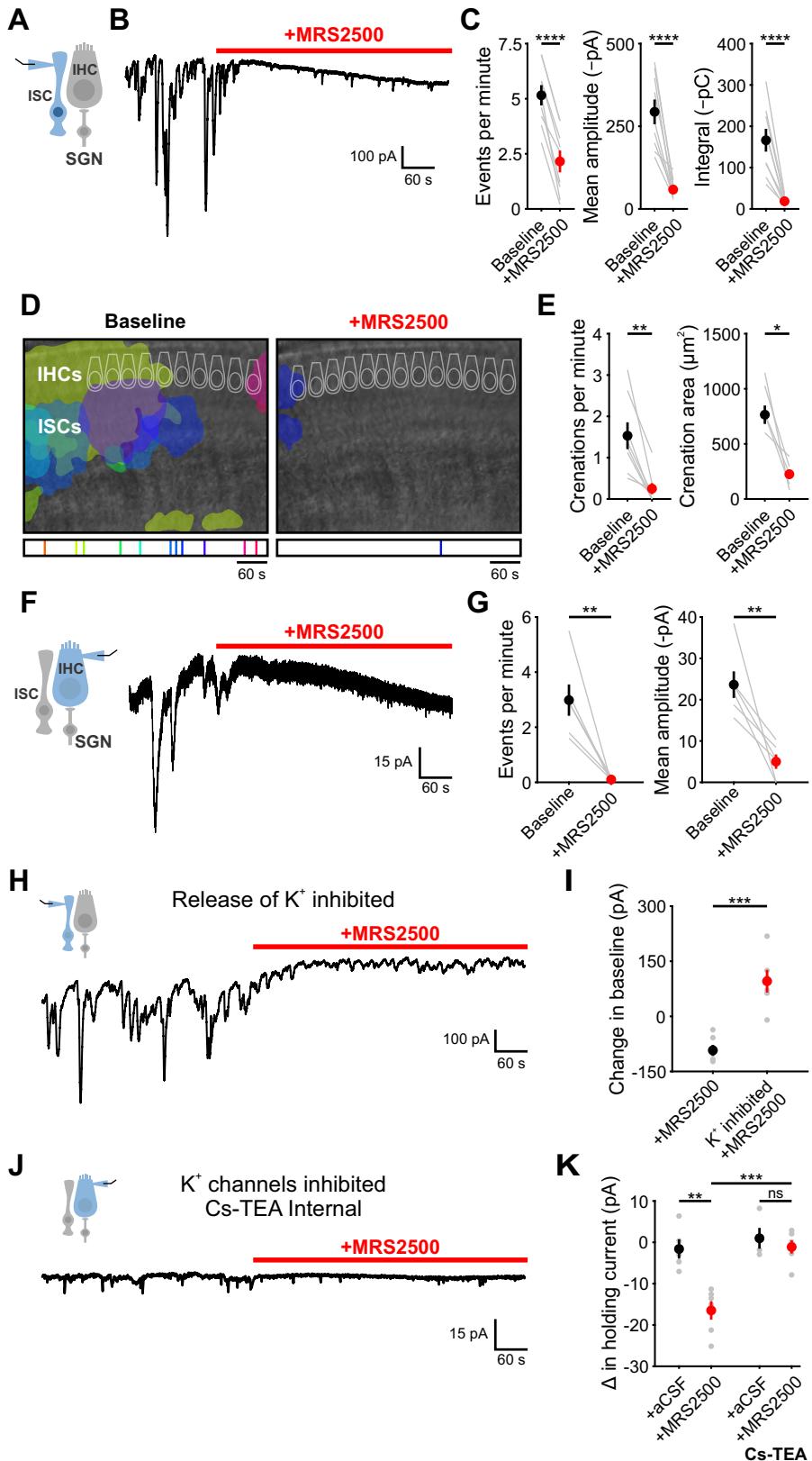


Figure 3. P2Y1 inhibition abolishes spontaneous currents in inner supporting cells and inner hair cells. **(A)** Schematic of ISC whole-cell recording configuration. **(B)** ISC spontaneous inward currents before and after application of MRS2500 (1 μ M). Recordings performed at \sim 25°C. **(C)** Plot of event frequency, amplitude, and integral (charge transfer) before and after MRS2500 application. $n = 8$ ISCs (two-tailed paired Student's t test; *** p <5e-5) **(D)** Intrinsic optical imaging performed before and after application of the P2RY1 antagonist, MRS2500 (1 μ M). Detected crenations are outlined in colors based on time of occurrence as indicated below image. Imaging performed at \sim 25°C. Caption continued on next page.

Figure 3. (E) Plot of crenation frequency and area before and after MRS2500 application. n = 7 cochleae (two-tailed paired Student's t test; **p<0.005) for frequency calculation and n = 5 cochleae (two-tailed paired Student's t test; *p<0.05) for area calculation. Cochleae that did not crenate in MRS2500 were excluded from area calculations. **(F)** Schematic of whole-cell recording configuration from IHCs. (right) Whole-cell voltage clamp recording from an IHC. **(G)** Plots of event frequency and amplitude before and after MRS2500 application. n = 6 IHCs (two-tailed paired Student's t test with Bonferroni correction; **p<0.005, *p<0.05). **(H)** Whole-cell voltage clamp recording of an ISC with MRS2500 application following pre-incubation with CdCl₂ (100μM), TTX (1μM), ouabain (10μM), and bumetanide (50μM) to limit K⁺ release. **(I)** Plot of change in holding current, defined as the 95% percentile current for each period. n = 6 ISCs for each condition (two-tailed Student's t test; ***p<0.0005). **(J)** IHC whole-cell voltage clamp recording with Cs-TEA internal solution (to inhibit K⁺ channels) before and after MRS2500 application. **(K)** Plot of the change in IHC holding current following control (aCSF) and MRS2500 with K-MeS and Cs-TEA internal. n = 5 IHCs for aCSF, n = 6 IHCs for MRS2500, n = 4 IHCs for aCSF with Cs-TEA internal, and n = 5 IHCs for MRS2500 with Cs-TEA internal (one-way ANOVA; ***p<0.005, **p<0.005, ns, not significant).

Figure 3-Figure supplement 1. P2RY1 inhibition abolishes spontaneous currents in inner supporting cells and inner hair cells near physiological temperature.

Figure 3-Figure supplement 2. Spontaneous inward currents and crenations are dramatically reduced in *P2ry1* KO mice.

133 by purinergic receptors (*Figure 3-Figure Supplement 1B-E*). Consistent with the involvement of
 134 P2RY1, the amplitude and total charge transfer of ISC events (*Figure 3-Figure Supplement 2A,B*)
 135 and size of spontaneous crenations (*Figure 3-Figure Supplement 2C,D*) were smaller in cochleae
 136 in *P2ry1* KO mice relative to controls. However, supporting cells *P2ry1* KO mice exhibited aberrant,
 137 gain-of-function activity consisting of frequent, small amplitude currents (*Figure 3-Figure Supple-*
 138 *ment 2A,B*), that were not blocked by MRS2500 or broad-spectrum P2 receptor antagonists (*Fig-*
 139 *ure 3-Figure Supplement 2E,F*).

140 ATP-mediated signaling in ISCs activates TMEM16A, triggering K⁺ efflux that depolarizes nearby
 141 IHCs. To assess whether P2RY1 signaling is also required for periodic excitation of IHCs prior to
 142 hearing onset, we assessed the sensitivity of spontaneous IHC inward currents to MRS2500 (*Fig-*
 143 *ure 3F*). Consistent with the supporting cell origin of IHC activity, application of MRS2500 (1μM) also
 144 abolished spontaneous currents in IHCs (*Figure 3F,G*). Together, these data suggest that P2RY1 is
 145 the primary purinergic autoreceptor on ISCs responsible for inducing periodic excitation of hair
 146 cells prior to hearing onset.

147 **P2RY1 inhibition leads to extracellular K⁺ accumulation**

148 Although P2RY1 inhibition abolished most transient inward currents in both ISCs and IHCs, a pro-
 149 gressively increasing inward current (downward shift in baseline) appeared in both cell types with
 150 prolonged application of MRS2500 (*Figure 3B,F*). Prior studies in CNS brain slices indicated that G_q-
 151 coupled purinergic receptors in astrocytes regulate extracellular K⁺ concentration and neuronal
 152 excitability (*Wang et al., 2012*). The slowly progressing nature of the response in IHCs and ISCs
 153 suggest that it may similarly arise from accumulation of K⁺ released from cells in the organ of Corti.
 154 If this hypothesis is correct, then inhibiting the main sources of K⁺ should diminish this inward
 155 current. Indeed, when IHC and SGN excitation was inhibited with tetrodotoxin (TTX, 1μM) and cad-
 156 mium (CdCl₂, 100μM), and the K⁺ transporters, Na,K-ATPase and NKCC, were inhibited with ouabain
 157 (10μM) and bumetanide (50μM), no inward current was induced in ISCs upon blocking P2RY1 (*Fig-*
 158 *ure 3H,I*). Similarly, if K⁺ accumulation is responsible for the current in IHCs, it should be abolished
 159 when the ability of IHCs to detect changes in K⁺ is reduced. When whole cell recordings were per-
 160 formed from IHCs using an internal solution containing Cs⁺ and TEA, which blocks most IHC K⁺
 161 channels (*Kros et al., 1998; Marcotti et al., 2003*), MRS2500 also did not induce an inward current
 162 (*Figure 3J,K*). Together, these results suggest that P2RY1 has two distinct effects in the cochlea; it
 163 induces the transient inward currents that triggers IHC burst firing and it accelerates the clearance
 164 of K⁺ within the organ of Corti.

165 To directly assess the relationship between P2RY1 activity and extracellular K⁺ accumulation
 166 near IHCs, we monitored K⁺ levels in the extracellular space using IHC K⁺ channels. Focal P2RY1
 167 stimulation with a selective agonist (MRS2365, 10μM), which mimics the effect of endogenous ATP
 168 by eliciting an inward current and crenations in ISCs in control but not *P2ry1* KO mice (*Figure 4A-C*),

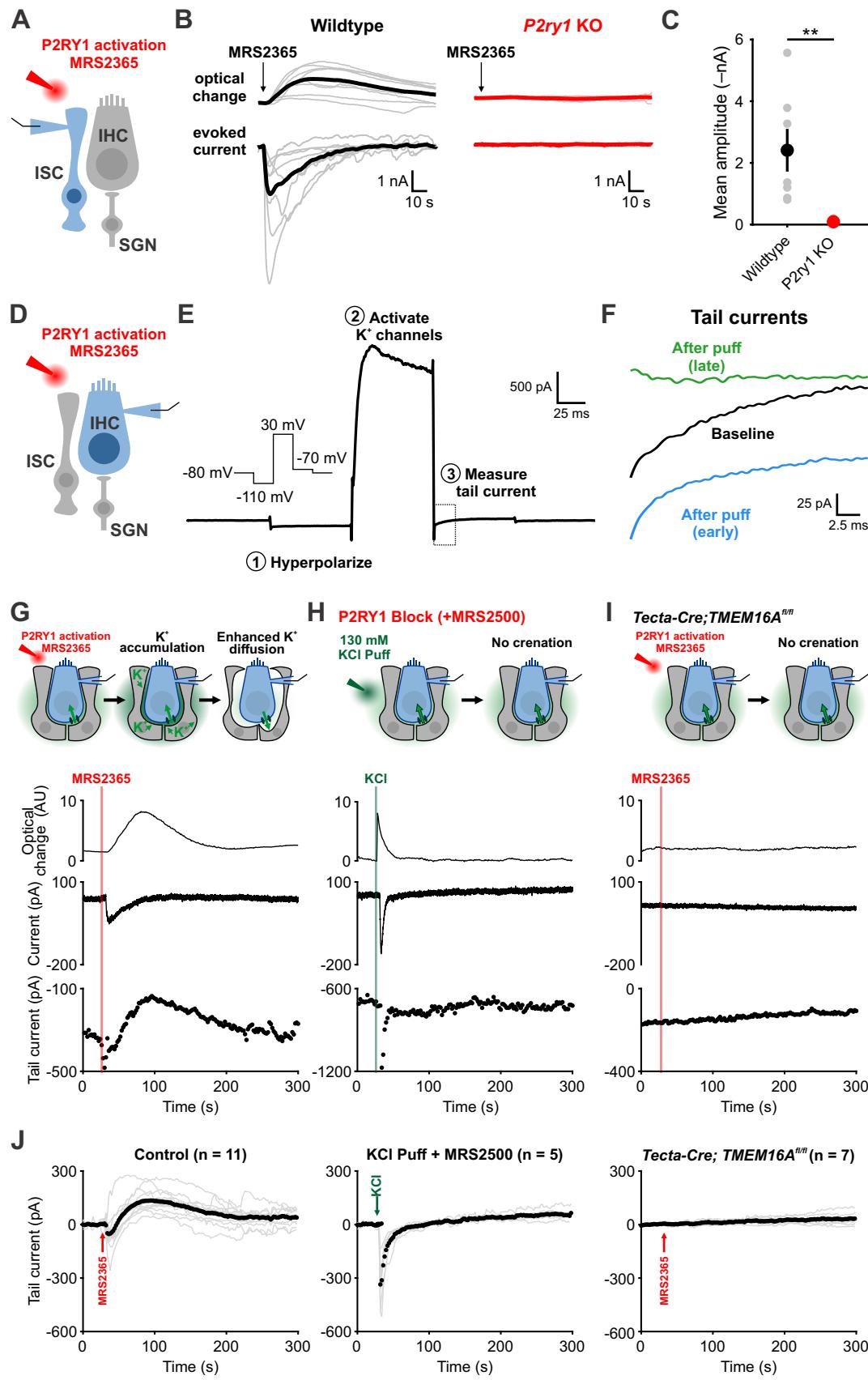


Figure 4. Caption on next page.

Figure 4. Activation of P2RY1 results in an initial accumulation of extracellular K⁺, followed by crenation and enhanced K⁺ clearance. **(A)** Schematic of whole-cell recording configuration from ISCs with puffs of MRS2365 (10μM), a P2RY1 agonist. **(B)** Optical change (crenation) and current elicited with MRS2365 puffs in wildtype and *P2ry1* KO mice. **(C)** Plot of mean current amplitude with MRS2365 puffs. n = 6 and n = 5 ISCs from wildtype and *P2ry1* KO mice, respectively (two-tailed Student's t test; **p<0.005). **(D)** Schematic of whole-cell recording configuration from IHCs with puffs of MRS2365 (10μM). **(E)** Example current trace and voltage-protocol designed to measure K⁺ accumulation. Dashed box indicated tail current measurement period indicated in (F). **(F)** Tail currents observed during baseline, immediately following the MRS2365 puff (with 2 seconds), and after the puff (30 seconds). **(G)** Model of K⁺ dynamics following MRS2365 stimulation. Initially, extracellular K⁺ rapidly increases following stimulation, but ISCs crenate, increasing the amount of extracellular space and K⁺ buffering. (bottom) Optical change (crenation), holding current, and tail current as a function of time with respect to MRS2365 puff. **(H)** Similar to G, but with KCl puffs (130μM) in cochlea treated with MRS2500. **(I)** Similar to G, but in *Tecta-Cre;TMEM16A^{fl/fl}* mice where TMEM16A has been conditionally removed from the sensory epithelium (see *Figure 4-Figure Supplement 1*). No crenations were observed with MRS2365 stimulation. **(J)** Plot of tail currents over time following MRS2365 stimulation. Grey lines indicate individual IHCs; black points indicate the mean across the population. Baseline was normalized to 0 pA for all traces.

Figure 4-Figure supplement 1. Crispr-Cas9 mediated generation of the *Tecta-Cre* mouse line.

169 was combined with assessments of the reversal potential of K⁺ currents in IHCs using a voltage
 170 protocol similar to one used to assess extracellular K⁺ buildup at vestibular calyceal synapses (*Lim*
 171 *et al., 2011*). This protocol consisted of: (1) a hyperpolarizing step to -110mV to relieve K⁺ chan-
 172 nel inactivation, (2) a depolarizing step to +30mV to activate outward K⁺ currents, and (3) a step
 173 to -70mV to obtain a “tail” current (*Figure 4D-F*). Because the conductance during this last step
 174 is largely mediated by K⁺ channels, it is highly sensitive to shifts in K⁺ driving force induced by
 175 changes in extracellular K⁺ (*Contini et al., 2017; Lim et al., 2011*). Following transient stimulation
 176 of P2RY1, K⁺ tail currents immediately shifted inward, as would be expected if extracellular K⁺ in-
 177 creases (*Figure 4G,I*), and is similar to the effects of a metabotropic purinergic receptor agonist
 178 (UTP) on synaptically-evoked K⁺ currents in IHCs (*Wang et al., 2015*). However, after a few seconds
 179 these K⁺ currents shifted outward relative to baseline, indicative of a gradual decrease in extracellu-
 180 lar K⁺ below that present prior to P2RY1 stimulation, then gradually returned to the pre-stimulation
 181 level after several minutes (*Figure 4G,I*).

182 The outward shift in K⁺ tail current followed the time course of the crenation ($\tau_{\text{decay}} = 100 \pm 14\text{s}$
 183 for tail currents and $\tau_{\text{decay}} = 38 \pm 4\text{s}$ for crenations), suggesting that the shrinkage of cells induced
 184 by P2RY1 activation results in a prolonged increase in extracellular space that may allow greater
 185 dilution and more rapid redistribution of K⁺ in the organ of Corti. Alternatively, buildup of extra-
 186 cellular K⁺ alone may stimulate greater uptake. To determine if rapid increases in extracellular
 187 K⁺ or Cl⁻ were sufficient to stimulate K⁺ redistribution in the absence of crenation, we puffed KCl
 188 (130mM) into the supporting cell syncytium in the presence of P2RY1 antagonists (*Figure 4H*). As
 189 expected, this transient increase in extracellular K⁺ induced an inward shift in K⁺ tail currents and
 190 a brief optical change induced by fluid delivery; however, K⁺ tail currents rapidly returned to base-
 191 line and did not shift outward, suggesting that K⁺ (and Cl⁻) efflux are not sufficient to enhance K⁺
 192 redistribution rates. In addition, we transiently stimulated P2RY1 in *Tecta-Cre;TMEM16A^{fl/fl}* mice, in
 193 which purinergic receptor activation is preserved, but crenations are abolished (*Wang et al., 2015*).
 194 In these mice, ISCs failed to crenate, IHCs did not depolarize, and K⁺ tail currents remained stable
 195 throughout the duration of the recording (*Figure 4I*). These results suggest that purinergic auto-
 196 receptors on ISCs influence extracellular K⁺ levels by both triggering K⁺ release and by altering K⁺
 197 redistribution by controlling the size of the extracellular space.

198 **P2ry1 mediates coordinated neuronal activation and precise burst firing of SGNs**

199 To evaluate the role of P2RY1 in initiating coordinated cellular activity in the cochlea, we monitored
 200 large-scale activity patterns in excised cochlea from *Pax2-Cre;R26-IsL-GCaMP3* mice, which express
 201 GCaMP3 in nearly all cells of the inner ear. Time lapse imaging revealed that the spontaneous Ca²⁺
 202 elevations that occur simultaneously within groups of ISCs, IHCs, and SGNs (*Tritsch and Bergles,*
 203 *2010; Zhang-Hooks et al., 2016*) were abolished following inhibition of P2RY1 with MRS2500 (*Fig-*
 204 *ure 5A-C*) and were dramatically reduced in *P2ry1* KO mice (*Pax2-Cre;R26-IsL-GCaMP3;P2ry1^{-/-}*) (*Fig-*

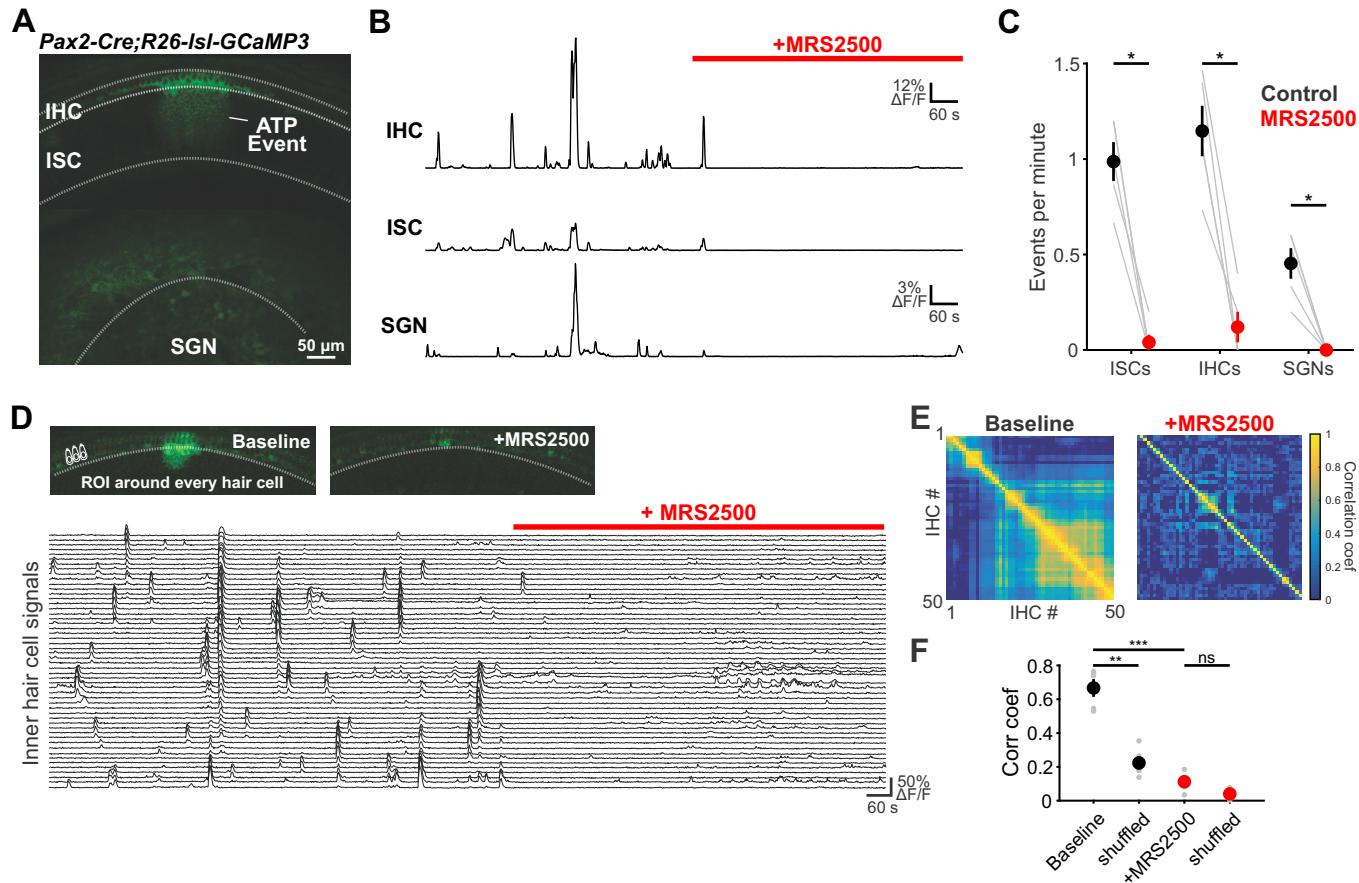


Figure 5. Large-scale coordinated activity in the cochlea requires P2RY1. **(A)** Exemplar Ca^{2+} transient in excised cochlea from *Pax2-Cre;R26-*Isl*-GCaMP3* mice. Note coordinated activation of ISCs, IHCs, and SGNs. **(B)** Traces of fluorescence intensity over time taken from ROIs that span the entire IHC, ISC, and SGN regions indicated in (A). **(C)** Plot of event frequency before and during application of MRS2500 (1 μM). $n = 5$ cochlea (two-tailed paired Student's t test with Bonferroni correction; * $p < 0.05$). **(D)** Exemplar images of IHC Ca^{2+} transients. ROIs were drawn around every IHC for subsequent analysis (bottom). **(E)** Correlation matrices generated by calculating the linear correlation coefficient for all IHC pairs before and after MRS2500 application. **(F)** Plot of average correlation coefficient calculated between the four nearest IHCs or four randomly shuffled IHCs. $n = 5$ cochleae (two-tailed paired Student's t test with Bonferroni correction; *** $p < 0.0005$, ** $p < 0.005$, ns, not significant).

Figure 5-Figure supplement 1. *P2ry1* KO mice exhibit reduced Ca^{2+} transients in ISCs.

ure 5–Figure Supplement 1A,B). Moreover, in accordance with the progressive increase in extracellular K⁺ that follows P2RY1 inhibition, there was a gradual increase in spontaneous, uncoordinated Ca²⁺ transients in IHCs in the presence of MRS2500 (Figure 5D–F), suggesting that this K⁺ accumulation increases IHC firing. Similarly, IHCs in *P2ry1* KO mice displayed a higher level of uncorrelated hair cell Ca²⁺ transients (Figure 5–Figure Supplement 1C–E), indicative of enhanced excitability. Together, these results indicate that P2RY1 is required for coordinated activation of ISCs, IHCs, and SGNs before hearing onset and that P2RY1 inhibition leads to higher rates of uncorrelated activity.

IHCs in the developing cochlea exhibit regenerative Ca²⁺ spikes that strongly activate post-synaptic SGNs, resulting in bursts of action potentials that propagate to the CNS. To determine if P2RY1 initiates burst firing in SGNs, we recorded spontaneous activity from SGNs using juxtapacellular recordings from their somata (Figure 6A). Application of MRS2500 resulted in a dramatic reduction of high frequency burst firing in SGNs, visible as a decrease in burst frequency and action potentials per burst (Figure 6E,F). All SGN spiking was abolished by the AMPA receptor antagonist NBQX (50μM) (Figure 6D), indicating that their activity requires synaptic excitation by IHCs. The precise patterning of action potentials within bursts was also disrupted by P2RY1 inhibition, as there were fewer interspike intervals in the 75–125ms range (Figure 6C,F), which correspond to the maximum rate of Ca²⁺ spike generation by IHCs during ATP-mediated excitation (Tritsch et al., 2010a). Additionally, the coefficient of variation measured for interspike intervals was significantly lower following P2RY1 inhibition, suggesting SGNs fire more randomly (Figure 6E). However, the average frequency of action potentials remained unchanged during P2RY1 inhibition (Figure 6E), due to increases in non-burst firing (Figure 3F,Figure 4D). SGNs in *P2ry1* KO cochleae exhibited activity similar to wildtype SGNs in the presence of MRS2500, with a lower burst firing rate, fewer interspike intervals in the 75–125ms range, and a lower coefficient of variation of interspike intervals relative to controls (Figure 6G–I). However, despite the profound contribution of P2RY1 to ISC and IHC activity, some burst-like behavior was still observed in SGNs (Figure 6D,G), suggesting that other forms of excitation emerge in the absence of P2RY1, due to an increase in overall excitability or developmental changes. Together, these data indicate that P2RY1 is required to generate discrete bursts of action potentials in SGNs and that loss of these receptors enhances uncorrelated firing.

234 P2RY1 promotes auditory neuron firing *in vivo*

The highly synchronized electrical activity exhibited by IHCs prior to hearing onset propagates through the entire developing auditory system to induce correlated firing of auditory neurons within isofrequency zones (Babola et al., 2018; Tritsch et al., 2010a). To determine if P2RY1 is required to produce this form of correlated activity, we used *in vivo* wide-field epifluorescence microscopy of the inferior colliculus (IC) in mice that express GCaMP6s in all neurons (*Snap25-T2A-GCaMP6s* and *Snap25-T2A-GCaMP6s;P2ry1*^{-/-} mice). Time lapse imaging revealed that both control and *P2ry1* KO mice exhibited correlated neuronal activity confined to stationary bands oriented along the tonotopic axis (Figure 7A–C). Spontaneous events were less frequent in *P2ry1* KO mice (9.7 ± 0.8 events per minute compared to 13.4 ± 0.7 events per minute in control; two-tailed Student's t test, p = 0.002), although the events were similar in amplitude and duration (half-width) (Figure 7D), suggesting that some compensatory amplification of events occurs in the CNS of these mice, similar to that seen in *Vglut3* KO mice (Babola et al., 2018). Spontaneous activity in *P2ry1* KO mice differed from controls in three other ways. First, the contralateral bias exhibited for each event was higher, with the weaker relative to stronger side amplitude decreasing from 0.61 ± 0.02 to 0.44 ± 0.02 (two-tailed Student's t test, p = 3.0e-6) (Figure 7D). Second, the coefficient of variation (ratio of standard deviation to the mean) of event amplitudes was 40% higher relative to controls (Figure 7D). Third, a detailed examination of the spatial location of events across the tonotopic axis (Figure 7E) revealed that activity in brain areas later responsible for processing higher frequency tones (~8 – 16 kHz) was reduced by 68% in *P2ry1* KO mice, while activity in low frequency areas was unaltered (Figure 7F–H). In *P2ry1* KO mice, bilateral removal of both cochleae abolished activ-

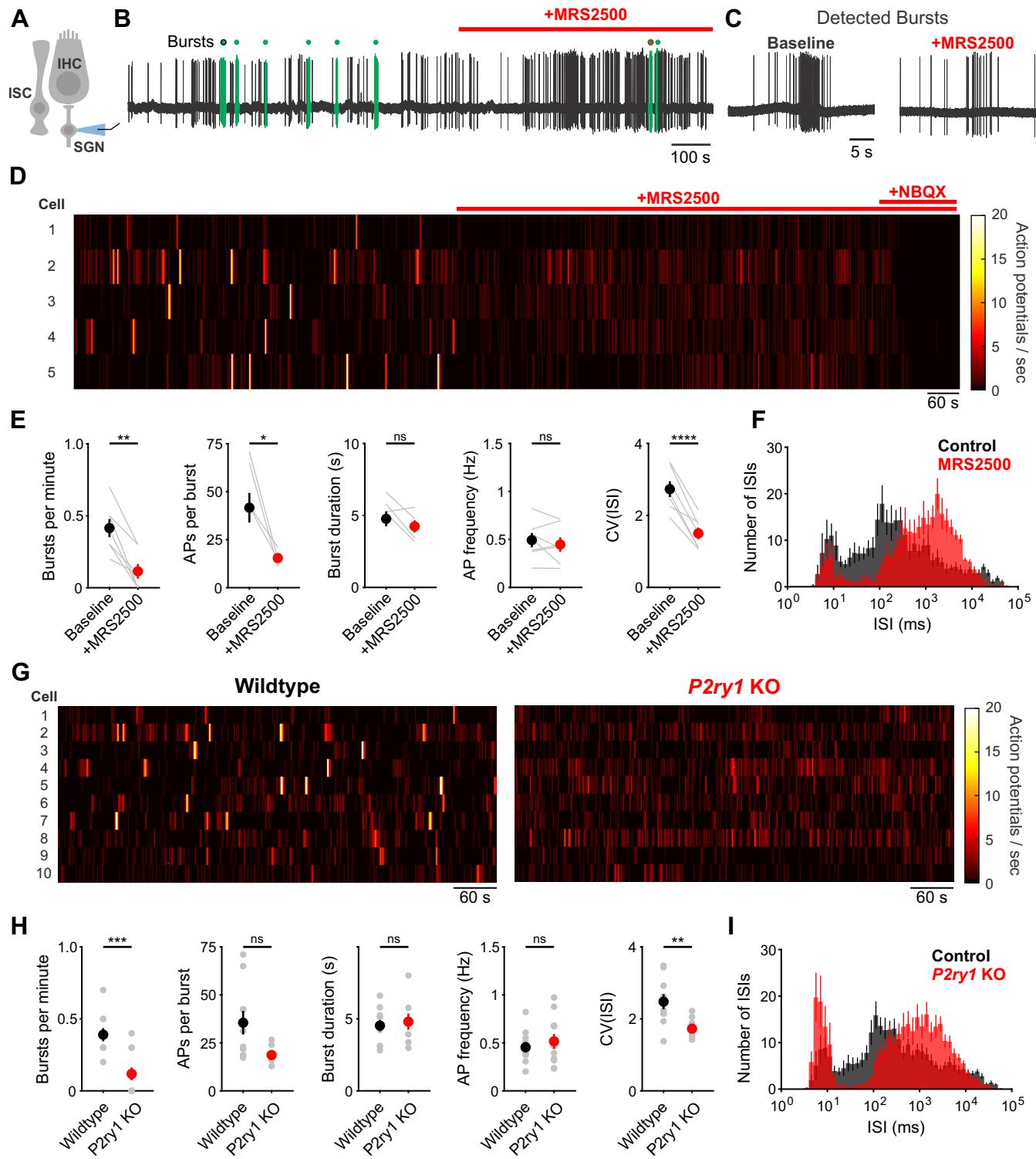


Figure 6. Inhibition of P2RY1 disrupts burst firing in spiral ganglion neurons. **(A)** Schematic of SGN juxtaglomerular recording configuration. All recordings were performed at ~25°C. **(B)** Action potentials recorded before and during MRS2500 application (1 μM). Detected bursts are indicated in green (see Methods and Materials for parameters used for burst detection). Circles with black and red outlines are expanded in **(C)**. **(C)** Action potentials within a detected burst before and after MRS2500 application. **(D)** Raster plots indicating the average firing rate of SGNs (bin: 1 s) before and after MRS2500 application (1 μM) and subsequent NBQX (50 μM). **(E)** Plots of average burst frequency, burst duration, action potentials (AP) per burst, average AP frequency, and coefficient of variation for all interspike intervals (ISIs) measured. $n = 7$ SGNs from 7 cochleae (two-tailed paired Student's t-test with Bonferroni correction; **** $p < 5e-5$, ** $p < 0.005$, * $p < 0.05$, ns, not significant). **(F)** Average log-binned interspike interval histograms before and after MRS2500 application. **(G)** Raster plots indicating the average firing rate of SGNs (bin: 1 s) in wildtype and *P2ry1* KO mice. **(H)** Plots of average burst frequency, burst duration, action potentials (AP) per burst, average AP frequency, and coefficient of variation for all ISIs measured. $n = 10$ wildtype and 11 *P2ry1* KO SGNs (two-tailed Student's t-test with Bonferroni correction; *** $p < 0.0005$, ** $p < 0.005$, ns, not significant). **(I)** Average log-binned interspike interval histograms from wildtype and *P2ry1* KO SGNs.

255 ity in the IC, demonstrating that activity in these mice originates in the periphery (**Figure 7–Figure**
 256 **Supplement 1A-C**).

257 Although *P2ry1* KO mice mimic some aspects of acute P2RY1 inhibition, the absence of P2RY1
 258 signaling throughout life may have led to compensatory changes, such as the increase in non-
 259 purinergic ISC activity (see **Figure 3–Figure Supplement 2E**). Therefore, to better assess the role
 260 of P2RY1 in generating spontaneous activity *in vivo*, we delivered a solution containing MRS2500
 261 into the intraperitoneal cavity of mice while imaging activity in the IC. Compared to mice injected
 262 with control solution (5% mannitol), mice injected with MRS2500 exhibited dramatic reductions in
 263 IC event frequency (from 13.3 ± 0.8 to 3.9 ± 1.1 events per minute; two-tailed Student's t test, $p =$
 264 0.0001) and amplitude (from 9.9 ± 0.5 to $4.9 \pm 0.8\%$ $\Delta F/F_0$; two-tailed Student's t test, $p =$ 0.0006)
 265 ~5 minutes after administration (**Figure 8A-D**). This decrease was specific to the IC, as SC retinal
 266 wave activity (**Ackman et al., 2012**) was unaffected by acute MRS2500 administration (**Figure 8B,C,E**),
 267 indicating that the locus of action is likely within the cochlea, which has been shown to have a less
 268 intact blood-tissue barrier at this age (**Suzuki et al., 1998**). Spatial analysis revealed that unlike the
 269 selective deficit observed in higher frequency zones in *P2ry1* KO mice, the inhibition was not limited
 270 to certain tonotopic regions, but rather occurred evenly across all frequency zones (**Figure 8F,G**).
 271 Together, these data indicate that ISC P2RY1 autoreceptors within the cochlea play a critical role in
 272 initiating spontaneous bursts of neural activity in auditory centers within the brain prior to hearing
 273 onset.

274 Discussion

275 Intense periods of neuronal activity dramatically alter the ionic composition of the extracellular
 276 environment, leaving behind excess K^+ that can alter neuronal excitability, induce spontaneous ac-
 277 tivity and trigger debilitating seizures. In the CNS, homeostatic control of extracellular K^+ levels is ac-
 278 complished by glial cells, which redistribute K^+ passively through ion channels and actively through
 279 facilitated transport, but much less is known about the mechanisms that control excitability in the
 280 peripheral nervous system. Sensory hair cells and primary auditory neurons in the cochlea are sur-
 281 rounded by supporting cells that share key features with CNS glia and are thought to redistribute
 282 K^+ that accumulates during sound detection. However, prior to hearing onset, ATP dependent K^+
 283 release from these cells triggers periodic bursts of activity in nearby IHCs that propagate through-
 284 out the auditory system. Here, we demonstrate that this form of intrinsically generated activity is
 285 initiated through activation of P2RY1, a G_q -coupled metabotropic purinergic receptor. Acute inhibi-
 286 tion or genetic removal of this receptor dramatically reduced spontaneous activity and disrupted
 287 burst firing in IHCs, SGNs and central auditory neurons. In addition to triggering episodic K^+ de-
 288 pendent depolarization of hair cells, activation of P2RY1 also enhanced K^+ clearance by increasing
 289 the volume of extracellular space, allowing more rapid dissipation of extracellular K^+ transients.
 290 This duality of purpose, to induce K^+ efflux and enhance K^+ clearance, promotes discrete bursts of
 291 activity throughout the developing auditory system.

292 Purinergic signaling in the developing cochlea

293 Before the onset of hearing, neurons in the auditory system that will process similar sound frequen-
 294 cies exhibit periodic bursts of highly correlated activity, an entrainment that is initiated within the
 295 cochlea (**Babola et al., 2018; Clause et al., 2014; Sonntag et al., 2009; Tritsch et al., 2010a**). Within
 296 the developing cochlear epithelium, spontaneous release of ATP from ISCs activates purinergic
 297 receptors, triggering a rapid increase of intracellular Ca^{2+} , gating of TMEM16A Ca^{2+} -activated Cl^-
 298 channels, and subsequent Cl^- and K^+ efflux into the extracellular space (**Tritsch et al., 2007; Wang**
 299 **et al., 2015**). This transient K^+ efflux is sufficient to depolarize nearby IHCs, resulting in a burst of
 300 Ca^{2+} action potentials, release of glutamate, and suprathreshold activation of postsynaptic SGNs
 301 via AMPA and NMDA receptors (**Tritsch et al., 2010a; Zhang-Hooks et al., 2016**). Our results show
 302 that activation of metabotropic P2RY1 autoreceptors is a key first step in this transduction pathway.
 303 P2RY1 is highly expressed by ISCs at a time when spontaneous activity is prominent in the cochlea

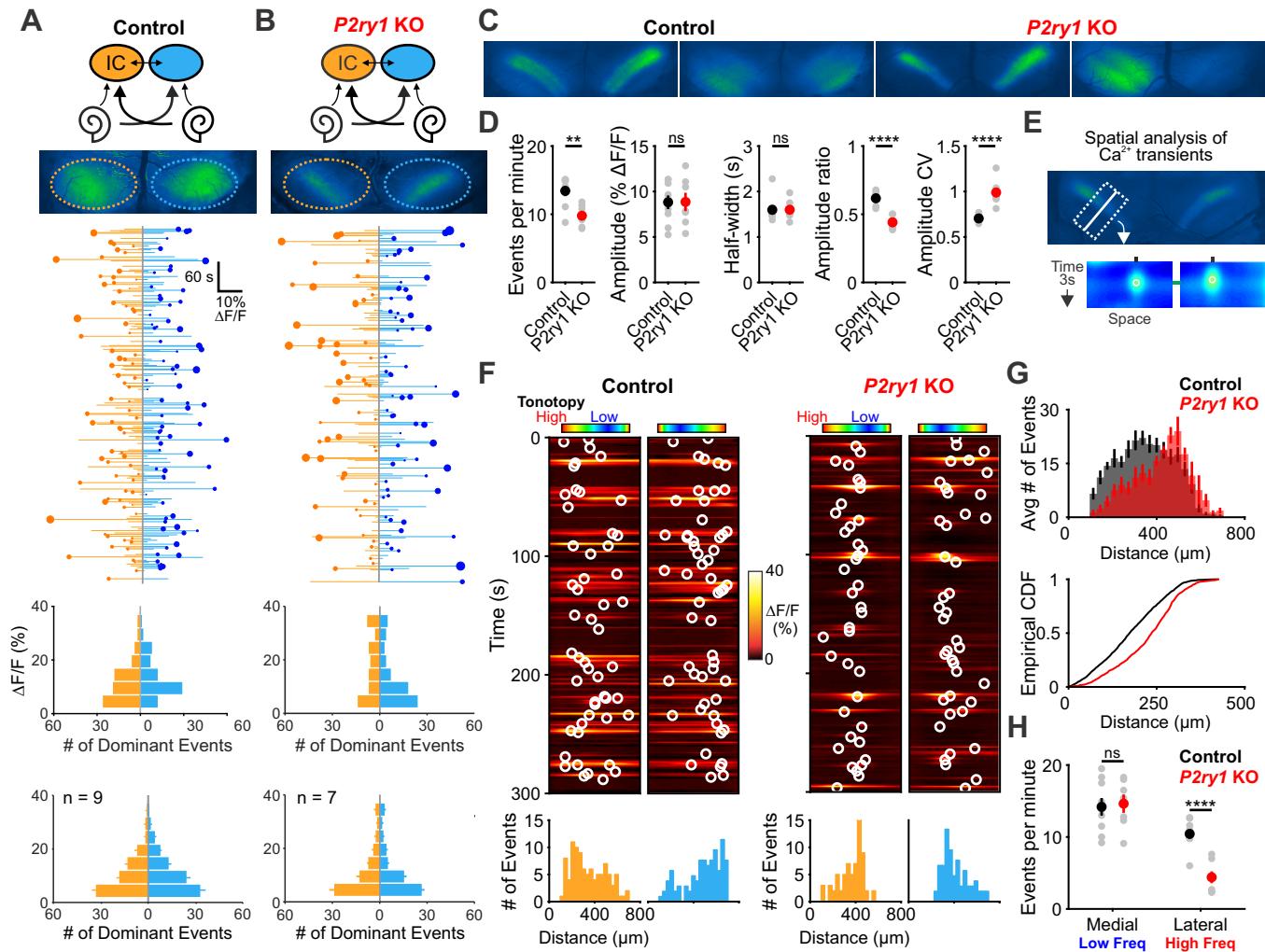


Figure 7. *P2ry1* KO mice exhibit reduced and spatially restricted spontaneous activity in the inferior colliculus. **(A)** Diagram illustrating flow of information through the auditory system and average intensity image over the 10 min imaging session. (middle) Activity over time in left and right IC in an individual where each line indicates the fluorescence intensity of each detected event; the circle indicates the dominant lobe, and the size of the circle indicated the difference in fluorescence. (bottom) Histograms showing the frequency of dominant events of a given amplitude for this experiment and for all experiments. Imaging was performed in *Snap25-T2A-GCaMP6s* mice ($n = 9$ mice). **(B)** Similar to (A), but in *Snap25-T2A-GCaMP6s;P2ry1^{-/-}* (*P2ry1* KO) mice ($n = 7$ mice). **(C)** Images of spontaneous events in the IC of in control (*Snap25-T2A-GCaMP6s*) and *P2ry1* KO mice (*Snap25-T2A-GCaMP6s;P2ry1^{-/-}*). **(D)** Comparisons of average frequency, amplitude, half-width, and event ratio from control and *P2ry1* KO mice. Bilateral amplitude ratio was calculated for events simultaneous across both lobes of the IC and defined as the ratio of the weak to the strong side amplitude. A ratio of 1 would indicate complete synchrony between lobes; a ratio of 0 would indicate complete asymmetry. $n = 9$ control and $n = 7$ *P2ry1* KO mice (two-tailed Student's t test with Bonferroni correction; **** $p < 5e-4$, ** $p < 0.005$, ns: not significant). **(E)** Exemplars of a single-banded event. Rectangular ROIs were placed as shown and averaged to create a 'line-scan' across the tonotopic axis. (bottom) Heat maps of activity as a function of time and distance; circles indicate detected peaks. **(F)** Activity over a five-minute time frame in the left and right IC of control and *P2ry1* KO mice. Circles indicate detected peaks. (bottom) Histograms of peak locations. **(G)** Histogram of average number of events across all control (black) and *P2ry1* KO (red) mice. (bottom) Cumulative distribution function of event locations across the tonotopic axis pooled from all animals. Events from left and right IC were combined for each experiment. **(H)** Quantification of event frequency in the medial (low frequency) and lateral (high frequency) regions of the IC. $n = 9$ control and 7 *P2ry1* KO mice (two-tailed Student's t test with Bonferroni correction; **** $p < 5e-5$, ns, not significant).

Figure 7-Figure supplement 1. Spontaneous activity in *P2ry1* KO mice originates in the cochlea.

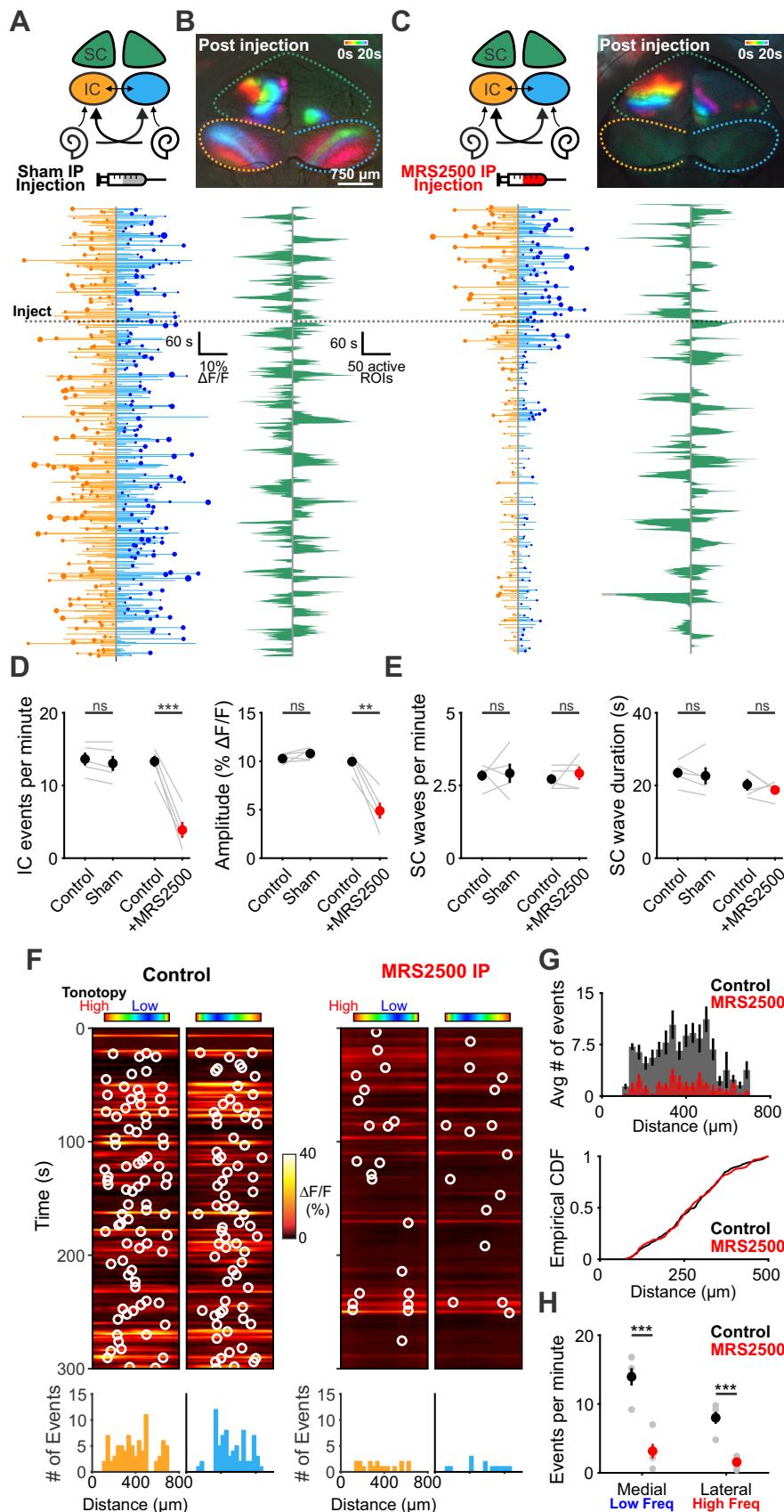


Figure 8. Delivery of MRS2500 *in vivo* dramatically reduces spontaneous activity in the developing auditory system. **(A)** Diagram illustrating flow of information to the midbrain and the visual superior colliculus. Sham solution (5% mannitol) was injected via IP catheter during imaging. (bottom) Activity over time in left and right IC in an individual. Each line indicates a detected event, circle indicates the dominant lobe, and the size of the circle indicates the difference in fluorescence. Dashed line is time of injection. Caption continued on next page.

Figure 8. **(B)** Calcium transients in the midbrain, color-coded based on time of occurrence following sham injection. (bottom) Calcium transients observed in the left and right SC. **(C)** Similar to (A) and (B), but with injection of MRS2500 (50 μ L of 500 μ M MRS2500 in 5% mannitol solution). **(D)** Plot of IC event frequency and amplitude in sham and MRS2500 injected animals. n = 5 mice for each condition (two-tailed paired Student's t test with Bonferroni correction; ***p<0.005, **p<0.005, ns: not significant). **(E)** Plot of SC wave frequency and duration in sham and MRS2500 injected animals. n = 5 mice for each condition (two-tailed paired Student's t test with Bonferroni correction; ns: not significant). **(F)** Activity along the tonotopic axis over a five-minute time frame in the left and right IC before (left) and after (right) MRS2500 injection. Circles indicate detected peaks. (bottom) Histograms of peak locations. **(G)** Histogram of average number of events before (black) and after (red) MRS2500 injection. (bottom) Cumulative distribution function of event locations across the tonotopic axis pooled from all animals. Events from left and right IC were combined for each experiment. **(H)** Quantification of event frequency in the medial (low frequency) and lateral (high frequency) regions of the IC. n = 5 mice (two-tailed Student's t test with Bonferroni correction; ***p<0.005).

304 (*Scheffer et al., 2015; Tritsch and Bergles, 2010*) (Figure 2A), and spontaneous activity was reduced
 305 when intracellular Ca²⁺ stores were depleted or PLC was inhibited (Figure 1B-F), manipulations that
 306 disrupt canonical G_q-coupled GPCR signaling pathways (*Erb and Weisman, 2012; Fabre et al., 1999*).
 307 Moreover, our pharmacological studies indicate that P2RY1 is both necessary and sufficient for
 308 spontaneous current generation in supporting cells (Figure 3B, Figure 4B), and inhibition of P2RY1
 309 *in vivo* profoundly decreased cochlea-generated activity in the auditory midbrain (Figure 8C). This
 310 reliance on P2RY1 is unexpected, as ionotropic P2X receptors are also widely expressed in the de-
 311 veloping cochlea (*Brändle et al., 1999; Lahne and Gale, 2008; Liu et al., 2015; Nikolic et al., 2003;*
 312 *Scheffer et al., 2015; Tritsch et al., 2007*). The lack of P2X engagement may reflect the particu-
 313 lar spatial-temporal characteristics of ATP release by ISC, which may occur in locations devoid of
 314 P2X receptors or yield ATP concentration transients that favor P2RY1 activation. Exogenous ATP
 315 can induce all of the phenomenon associated with spontaneous events (ISC currents, crenation,
 316 IHC depolarization, SGN burst firing); however, it is possible that other nucleotides are released
 317 that have greater affinity for P2RY1 (e.g. ADP), or that extracellular nucleotidases rapidly convert
 318 ATP to ADP/AMP that favor activation of these native metabotropic receptors (*von Kügelgen, 2006;*
 319 *Vlajkovic et al., 1998, 2002*).

320 Control of extracellular K⁺ dynamics by supporting cells

321 Pharmacological inhibition of P2RY1 unexpectedly induced IHCs to gradually depolarize and begin
 322 tonic, uncorrelated firing, a phenotype also observed in *P2ry1* KO mice (Figure 5-Figure Supple-
 323 ment 1C). Our studies indicate that this phenomenon occurs because P2RY1 controls the volume
 324 of the extracellular space in the organ of Corti. Activation of P2RY1 induces ISCs to shrink osmot-
 325 ically (crenate), a consequence of ion and water efflux that is triggered by opening of TMEM16A
 326 channels (Figure 4G). The resulting increase in extracellular space lasts for many seconds and en-
 327 hances dissipation of extracellular K⁺, visible through the time-dependent shift in the reversal po-
 328 tential of K⁺ mediated tail currents (Figure 4G,I). Conversely, inhibition of P2RY1 increased the size
 329 of ISCs, a swelling-induced “relaxation” that concomitantly decreased extracellular space around
 330 IHCs. K⁺ accumulation and depolarization of IHCs followed, an effect absent when IHC K⁺ channels
 331 were inhibited (Figure 3J,K). This phenomenon is consistent with the depolarizing shift in the resting
 332 membrane potential of IHCs observed in *Tmem16A* cKO mice (*Wang et al., 2015*), which similarly
 333 blocks ISC crenation, and with studies in the brain where inducing cell swelling with hypoosmotic
 334 solutions or impairing K⁺ buffering results in neuronal epileptiform activity (*Larson et al., 2018;*
 335 *Murphy et al., 2017; Thrane et al., 2013*). Basal P2RY1 activation in supporting cells therefore hy-
 336 perpolarizes nearby IHCs in the developing cochlea by expanding the extracellular space and low-
 337 ering local K⁺ concentrations. These changes increase the dynamic range of IHCs and allow finer
 338 control of excitability through transient ATP mediated signaling events.

339 The tonic inward current that develops in ISCs in response to P2RY1 block was abolished when
 340 homeostatic K⁺ release pathways (Na⁺ channels, Ca²⁺ channels, Na⁺-K⁺-Cl⁻cotransporters, and Na,K-
 341 ATPase) were inhibited (Figure 3H,I), suggesting that K⁺ redistribution mechanisms, in the absence
 342 of ISC crenation, are weak at this stage of development. Indeed, although the membrane poten-

343 tial of ISCs is close to E_K , their membrane conductance is dominated by intercellular gap junction
 344 channels; when uncoupled from their neighbors, they exhibit very high ($1-2G\Omega$) input resistance
 345 (Jagger and Forge, 2014; Wang et al., 2015), suggesting that few K^+ leak channels are expressed.
 346 The presence of tight junctions at the apical surface of the cochlear epithelium and the limited K^+
 347 conductance of ISCs may restrict passive diffusion and dilution of K^+ , similar to what has been de-
 348 scribed in the vestibular epithelium (Contini et al., 2017), thus necessitating uptake via alternative
 349 mechanisms. Both inner phalangeal and Dieters' cells (which envelop the inner and outer hair cells,
 350 respectively) express K^+-Cl^- symporters, Na,K-ATPase pumps, and inwardly-rectifying K^+ channels
 351 that may siphon K^+ into the supporting cell syncytium after extrusion from hair cells. However,
 352 the apparently low capacity of these systems places a greater dependence on diffusion within the
 353 extracellular volume fraction controlled by the supporting cells.

354 In the CNS, astrocytes facilitate rapid dissipation of accumulated K^+ through the glial syncytium
 355 via gap junctions (Kofuji and Newman, 2004), a mechanism termed spatial buffering. Astrocytes
 356 are efficient K^+ sinks due to their highly negative resting potential (~ -85 mV) and large resting K^+
 357 conductance dominated by inward rectifying K^+ channels and two-pore K^+ channels (Ryoo and
 358 Park, 2016; Olsen, 2012). While uptake of K^+ through these conductances is passive, recent studies
 359 suggest that K^+ buffering in astrocytes is also actively regulated by purinergic receptors. Following
 360 stimulation of native astrocyte purinergic receptors or foreign G_q-coupled receptors (MrgA1) and
 361 release of Ca^{2+} from intracellular stores, Na,K-ATPase activity increased, resulting in a transient
 362 decrease in extracellular K^+ , hyperpolarization of nearby neurons, and reduction in their sponta-
 363 neous activity (Wang et al., 2012). Although P2RY1 is expressed by some astrocytes and can trigger
 364 Ca^{2+} waves (Gallagher and Salter, 2003), this mechanism does not appear to regulate IHC excitabil-
 365 ity in the cochlea, as stimulation of P2RY1 in *Tmem16a* cKO mice, which have intact metabotropic
 366 receptor signaling but no crenations (Wang et al., 2015), did not hyperpolarize IHCs (Figure 4I,J).
 367 Thus, astrocytes and cochlear ISCs use purinergic signaling in different ways to maintain the ionic
 368 stability of the extracellular environment and control the excitability of nearby cells.

369 **Role of supporting cells in the generation of spontaneous activity**

370 Our understanding of how non-sensory cells contribute to spontaneous activity has been limited by
 371 a lack of *in vivo* mechanistic studies. Recent advances in visualizing cochlea-induced spontaneous
 372 activity in central auditory centers *in vivo* using genetically-encoded calcium indicators (Babola
 373 et al., 2018) allowed us to assess whether supporting cell purinergic receptors are involved in gen-
 374 erating this activity. Prior to hearing onset, the blood-labyrinth barrier within the inner ear is not
 375 fully formed (Suzuki et al., 1998), permitting pharmacological access to the cochlea at this age. In-
 376 fusion of a P2RY1 inhibitor into the intraperitoneal space dramatically decreased activity within
 377 the inferior colliculus within minutes, while retina-induced activity in the superior colliculus (Ack-
 378 man et al., 2012) was unaffected (Figure 8), suggesting that inhibition is not due to activation of
 379 astrocyte P2RY1 receptors; as noted above, inhibition of P2RY1 in astrocytes would be expected to
 380 enhance, rather than inhibit neuronal activity (Wang et al., 2012).

381 *In vivo* imaging in *P2ry1* KO mice recapitulated many aspects of changes seen when P2RY1 was
 382 acutely inhibited, with significantly reduced neuronal activity observed in lateral regions of the IC
 383 (later active to 8-16kHz tones; Figure 7). However, neuronal burst firing persisted within central
 384 regions of the IC, regions that will ultimately process lower frequency sounds (3-8kHz). Developing
 385 sensory systems exhibit a remarkable ability to preserve spontaneous activity. In the visual system,
 386 cholinergic antagonists injected directly into the eye blocks retinal waves *in vivo* (Ackman et al.,
 387 2012), but genetic removal of the $\beta 2$ acetylcholine receptor subunit alters, but does not abolish,
 388 peripherally-generated activity (Zhang et al., 2012). In the auditory system, *in vivo* spontaneous
 389 activity can be blocked by acute inhibition of cochlear AMPARs, but deaf mice that lack the ability
 390 to excite SGNs (*Vglut3* KO mice) exhibit activity patterns remarkably similar to control mice (Babola
 391 et al., 2018). These robust homeostatic mechanisms allow spontaneous activity to persist despite
 392 disruption of key transduction components. Local purinergic signaling within the cochlea may still

393 initiate tonotopic activity in central auditory circuits of *P2ry1* KO mice, as events in the IC exhibited
 394 spatial and temporal characteristics similar to controls. IHCs and SGNs are more depolarized in
 395 these mice, reducing the threshold for activation by other purinergic receptors. Although such
 396 gain-of-function changes in the developing nervous system present challenges for interpretation
 397 of genetic manipulations, preservation of early, patterned activity in children that carry deafness
 398 mutations may improve the outcome of later therapeutic interventions to restore hearing.

399 **Purinergic receptors in the adult cochlea**

400 In the adult inner ear, members of all purinergic receptors subtypes (ionotropic P2X receptors,
 401 metabotropic P2Y, and adenosine P1 receptors) are expressed by cells throughout the sensory ep-
 402 ithelium, Reissner's membrane, stria vascularis, and SGNs (*Housley et al., 2009; Huang et al., 2010*).
 403 The widespread expression of these receptors coupled with observations of increased endolym-
 404 phatic ATP concentrations following trauma (*Muñoz et al., 1995a*) have led to the hypothesis that
 405 these receptors serve a neuroprotective role. Indeed, infusion of ATP into the inner ear profoundly
 406 reduces sound-evoked compound action potentials in the auditory nerve (*Bobbin and Thompson,*
 407 *1978; Muñoz et al., 1995b*), due to decreased endolymphatic potential following shunting inhibi-
 408 tion through P2XR2 (*Housley et al., 2013*). Consistent with these observations, *P2rx2* KO mice and
 409 humans with a P2RX2 variant (c.178G > T) experience progressive sensorineural hearing loss (*Yan*
 410 *et al., 2013*). Ca²⁺ imaging and recordings from adult cochleae have also revealed robust responses
 411 to UTP in the inner sulcus, pillar cells, and Dieters' cells (*Sirko et al., 2019; Zhu and Zhao, 2010*),
 412 suggesting that metabotropic purinergic receptors continue to be expressed. Following traumatic
 413 noise damage, ATP release could activate K⁺ buffering mechanisms in supporting cells, enhance
 414 K⁺ redistribution, reduce IHC depolarization and prevent excitotoxic damage. Purinergic receptors
 415 may also contribute to IHC gain control by influencing their membrane potential, as ATP circulates
 416 in the endolymph at low nanomolar concentrations (*Muñoz et al., 1995a*). Further studies involv-
 417 ing conditional deletion of *P2ry1* from ISCs in the adult cochlea may help to define the role of this
 418 receptor in both normal hearing and injury contexts.

419 **Methods and Materials**

420 Both male and female mice and rats of postnatal days P6–P15 were used for all experiments and
 421 randomly allocated to experimental groups. Transgenic breeders were crossed to female FVB mice
 422 to improve litter survival. Mice were housed on a 12 hour light/dark cycle and were provided food
 423 ad libitum. This study was performed in accordance with the recommendations provided in the
 424 Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All experi-
 425 ments and procedures were approved by the Johns Hopkins Institutional Care and Use Committee
 426 (protocol M018M350). All surgery was performed under isoflurane anesthesia and every effort was
 427 made to minimize suffering.

428 **Electrophysiology**

429 For inner supporting cell recordings, apical segments of the cochlea were acutely isolated from
 430 P6-P8 rat (*Figure 1*) and mouse pups (all other figures) and used within 2 hours of the dissection.
 431 Cochleae were moved into a recording chamber and continuously superfused with bicarbonate-
 432 buffered artificial cerebrospinal fluid (1.5–2mL/min) consisting of the following (in mM): 119 NaCl,
 433 2.5 KCl, 1.3 MgCl₂, 1.3 CaCl₂, 1 NaH₂PO₄, 26.2 NaHCO₃, 11 D-glucose and saturated with 95% O₂ / 5%
 434 CO₂ to maintain a pH of 7.4. Solutions were superfused at either room temperature or near phys-
 435 iological temperature (32–34°C) using a feedback-controlled in-line heater (Warner Instruments),
 436 as indicated in figure legends. Whole-cell recordings of inner supporting cells (ISCs) were made
 437 under visual control using differential interference contrast microscopy (DIC). Electrodes had tip
 438 resistances between 3.5–4.5MΩ when filled with internal consisting of (in mM): 134 KCH₃SO₃, 20
 439 HEPES, 10 EGTA, 1 MgCl₂, 0.2 Na-GTP, pH 7.3. Spontaneous currents were recorded with ISCs held
 440 at -80mV.

441 For inner hair cell recordings, apical segments of the cochlea were acutely isolated from P6-
 442 P8 mouse pups and used within 2 hours of the dissection. Cochleae were moved into a recording
 443 chamber and continuously superfused with bicarbonate-buffered artificial cerebrospinal fluid (1.5-
 444 2mL/min) consisting of the following (in mM): 115 NaCl, 6 KCl, 1.3 MgCl₂, 1.3 CaCl₂, 1 NaH₂PO₄,
 445 26.2 NaHCO₃, 11 D-glucose. Solutions were saturated with 95% O₂ / 5% CO₂ to maintain a pH of
 446 7.4. Solutions were superfused at room temperature. Electrodes had tip resistances between 4.5-
 447 6.0MΩ when filled with internal consisting of (in mM): 134 KCH₃SO₃, 20 HEPES, 10 EGTA, 1 MgCl₂,
 448 0.2 Na-GTP, pH 7.3. For hair cell recordings with K⁺ channels inhibited with cesium and TEA, the
 449 internal solution consisted of (in mM): 100 cesium methanesulfonate, 20 TEA-Cl, 10 EGTA in CsOH,
 450 20 HEPES, 1 MgCl₂, 0.2 Na-GTP, pH 7.3 with CsOH. Spontaneous currents were recorded with IHCs
 451 held at near their resting membrane potential (-75 to -80mV).

452 Errors due to the voltage drop across the series resistance and the liquid junction potential
 453 were left uncompensated for recordings of spontaneous activity. For IHC recordings with K⁺ accu-
 454 mulation voltage protocols (Figure 4), the amplifier compensation circuit was used to compensate
 455 70% of the access resistance. Recordings that displayed more than a 10% increase in access re-
 456 sistance or access resistances > 30 MΩ were discarded. ISC and IHC spontaneous currents were
 457 recorded with pClamp 10 software using a Multiclamp 700B amplifier, low pass filtered at 2kHz,
 458 and digitized at 5kHz with a Digidata 1322A analog-to-digital converter (Axon Instruments).

459 Action potentials were analyzed offline using custom routines written in Matlab 2017b (Math-
 460 works). Briefly, raw traces were high-pass filtered to remove baseline drift and spikes were identi-
 461 fied using an amplitude threshold criterion. As described previously (*Tritsch et al., 2010a*), bursts
 462 were identified by classifying interspike intervals into non-bursting intervals (> 1s), burst intervals
 463 (30ms-1s), and mini-burst intervals (< 30ms). Bursts were defined as clusters of at least 10 con-
 464 secutive burst intervals (with mini-burst intervals being ignored in the context of burst detection).
 465 Spikes within mini-bursts were included when calculating the number of spikes within a burst. Col-
 466 ored raster plots were generated by grouping spikes into one-second bins and applying a color
 467 map to the resulting data (modified 'hot' colormap; Matlab).

468 **Cochlear explant culture**

469 Cochleae were dissected from postnatal day 5-6 control (*P2ry1*^{+/+} or *Pax2-Cre;R26-IsI-GCaMP3*) and
 470 *P2ry1* KO (*P2ry1*^{-/-} or *Pax2-Cre;R26-IsI-GCaMP3;P2ry1*^{-/-}) mice in ice-cold, sterile HEPES-buffered arti-
 471 ficial cerebrospinal fluid (aCSF) consisting of the following (in mM): 130 NaCl, 2.5 KCl, 10 HEPES,
 472 1 NaH₂PO₄, 1.3 MgCl₂, 2.5 CaCl₂, and 11 D-Glucose. Explants were mounted onto Cell-Tak (Corn-
 473 ing) treated coverslips and incubated at 37°C for 24 hours in Dulbecco's modified Eagle's medium
 474 (F-12/DMEM; Invitrogen) supplemented with 1% fetal bovine serum (FBS) and 10U/mL penicillin
 475 (Sigma) prior to recording or imaging.

476 **Transmitted light imaging**

477 Cochlear segments were imaged with a Olympus 40x water immersion objective (LUMPlanFl/IR)
 478 and recorded using MATLAB and a USB capture card (EZ Cap). Difference movies were generated
 479 by subtracting frames at time t_n and t_{n+5} seconds using ImageJ software to generate an index of
 480 transmittance change over time. To quantify transmittance changes, a threshold of three standard
 481 deviations above the mean was applied to the values. To calculate the frequency of these events,
 482 the whole field was taken as an ROI and peaks were detected using MATLAB (findpeaks function).
 483 To calculate area of these events, a Gaussian filter ($\sigma= 2.0$) was applied to the image after thresh-
 484 olding and the borders detected using MATLAB (bwlabel function). The area was then calculated as
 485 the number of pixels within the border multiplied by the area scaling factor ($\mu\text{m}/\text{pixel}$)² measured
 486 with a stage micrometer.

487 **Immunohistochemistry and X-gal Reaction**

488 Mice were deeply anesthetized with isoflurane and perfused with freshly prepared paraformalde-
 489 hyde (4%) in 0.1 M phosphate buffer. Cochleae were post-fixed for 45 minutes at room tempera-
 490 ture and stored at 4°C until processing. For X-gal reactions, P6-P8 cochleae were removed from
 491 the temporal bone and washed 3 x 5 minutes with PBS. Tissue was then incubated for 24 hours
 492 in the dark at 37°C in X-gal working solution consisting of (in mM): 5 K⁺ ferricyanide crystalline, 5
 493 K⁺ ferricyanide trihydrate, 2 magnesium chloride, and 0.1% X-gal (GoldBio) dissolved in DMSO. Af-
 494 ter washing 3 x 5 minutes with PBS, images of cochleae were acquired on a dissecting microscope
 495 (Zeiss Stemi 305). For immunohistochemistry, fixed tissue was washed 3 x 5 minutes in PBS, placed
 496 in 30% sucrose solution overnight, and incubated in OCT mounting medium overnight at 4°C. Ten
 497 micron thick cross-sections of the cochlea were made on a cryostat and mounted on Superfrost
 498 Plus slides (Fisher), which were then allowed to dry for 1 hour before processing. Cross-sections
 499 were incubated overnight with primary antibodies against β-gal (anti-Chicken; 1:4000, Aves) and
 500 Myosin-VIIa (anti-Rabbit; 1:500, Proteus BioSciences) for detection of β-gal and Myosin-VIIa only for
 501 qualitative analysis of the *Tecta-Cre;TdT* reporter mouseline (**Figure 4–Figure Supplement 1**). Sec-
 502 tions were then rinsed three times with PBS and incubated for two hours at room temperature with
 503 secondary antibodies raised in donkey (Alexa-488 and Alexa-546; 1:2000, Life Technologies). Slides
 504 were washed three times in PBS (second wash with PBS + 1:10,000 DAPI), allowed to dry, and sealed
 505 using Aqua Polymount (Polysciences, Inc.). Images were captured using a laser scanning confocal
 506 microscope (LSM 510 or 880, Zeiss).

507 **Confocal imaging of explants**

508 After one day *in vitro*, cochleae were moved into a recording chamber and continuously superfused
 509 with bicarbonate-buffered artificial cerebrospinal fluid (1.5 - 2mL/min) consisting of the following
 510 (in mM): 119 NaCl, 2.5 KCl, 1.3 MgCl₂, 1.3 CaCl₂, 1 NaH₂PO₄, 26.2 NaHCO₃, 11 D-glucose, and sat-
 511 urated with 95% O₂ / 5% CO₂ to maintain a pH of 7.4. A piezo-mounted objective was used to
 512 rapidly alternate between SGN cell bodies and ISCs/IHCs. Images were captured at 1 frame per
 513 second using a Zeiss laser scanning confocal microscope (LSM 710, Zeiss) through a 20X objective
 514 (Plan APOCHROMAT 20x/1.0 NA) at 512 x 512 pixel (354 x 354μm; 16-bit depth) resolution. Sec-
 515 tions were illuminated with a 488nm laser (maximum 25mW power). MRS2500 (1 μM, Tocris) was
 516 applied by addition to the superfusing ACSF.

517 **Analysis of *in vitro* Ca²⁺ transients**

518 Images were imported into ImageJ and image registration (MultiStackReg) was used to correct for
 519 drifts in the imaging field. Since images were obtained at two different z-planes, images were
 520 combined into one stack for analysis. This was done by eliminating the empty bottom half of the
 521 imaging field containing ISCs and IHCs and the empty top half of the field containing SGN cell bodies
 522 and merging the two images. For analysis of coordinated activity throughout the cochlea, regions
 523 of interest were drawn around the entirety of ISCs, IHCs, and SGNs. Fluorescence changes were
 524 normalized as ΔF/F₀ values, where ΔF = F - F₀ and F₀ was defined as the fifth percentile value for
 525 each pixel. Peaks in the signals were detected in MATLAB using the built-in peak detection function
 526 (findpeaks) with a fixed value threshold criterion (mean + 3 standard deviations for each cell).

527 To quantify frequency and areas of Ca²⁺ transients, a threshold of three standard deviations
 528 above the mean was applied to each pixel within the ROI. To calculate the frequency of these events,
 529 the whole field was taken as an ROI and peaks were detected using MATLAB (findpeaks function) on
 530 the number of thresholded pixels per frame. To calculate area of these events, a Gaussian filter ($\sigma=$
 531 2.0) was applied to the image after thresholding and the borders detected using MATLAB (bwlabel
 532 function). The area was then calculated as the number of pixels within the border multiplied by an
 533 area scaling factor (1 μm/pixel)² measured with a stage micrometer.

534 For correlation analysis, ROIs were drawn around every IHCs in the field of view. Pairwise cor-
 535 relation coefficients were performed between every hair cell pair and represented as correlation

536 matrices.

537 **Installation of cranial windows**

538 Inhalation anesthesia was induced with vaporized isoflurane (4% for 5 minutes, or until mice are
 539 non-responsive to toe-pinch) and surgical plane maintained during the procedure (with 1-2% isoflu-
 540 rane) with a stable respiration rate of 80 breaths per minute. A midline incision beginning posterior
 541 to the ears and ending just anterior to the eyes was made. Two subsequent cuts were made to
 542 remove the dorsal surface of the scalp. A headbar was secured to the head using super glue (Krazy
 543 Glue). Fascia and neck muscles overlying the interparietal bone were resected and the area bathed
 544 in sterile, HEPES-buffered artificial cerebrospinal fluid that was replaced as necessary throughout
 545 the surgery. Using a 28G needle and microblade, the sutures circumscribing the interparietal bone
 546 were cut and removed to expose the midbrain. The dura mater was removed using fine scissors
 547 and forceps, exposing the colliculi and extensive vasculature. A 5 mm coverslip (CS-5R; Warner In-
 548 struments) was then placed over the craniotomy, the surrounding bone was dried using a Kimwipe,
 549 and super glue was placed along the outer edges of the coverslip for adhesion to the skull. Replace-
 550 ment 0.9% NaCl solution was injected IP and a local injection of lidocaine was given to the back of
 551 the neck. Animals were weaned off isoflurane, placed under a warming lamp, and allowed to re-
 552 cover for a minimum of 1 hour prior to imaging. Spontaneous activity was not seen in deeply anes-
 553 thetized animals and emerged 30 minutes after recovery from isoflurane exposure, as reported
 554 previously (*Ackman et al., 2012*).

555 ***In vivo* calcium imaging**

556 After 1 hour of post-surgical recovery from anesthesia, pups were moved into a swaddling 15 mL
 557 conical centrifuge tube. The top half of this tube was removed to allow access to the headbar and
 558 visualization of the midbrain. Pups were head-fixed and maintained at 37°C. using a heating pad
 559 and temperature controller (TC-1000; CWE). During the experiments, pups were generally immo-
 560 bile; however, occasional limb and tail twitching did occur.

561 For wide field epifluorescence imaging, images were captured at 10 Hz using a Hamamatsu
 562 ORCA-Flash4.0 LT digital CMOS camera attached to a Zeiss Axio Zoom.V16 stereo zoom microscope.
 563 For midbrain imaging, a 4 x 4mm field of view was illuminated continuously with a mercury lamp
 564 (Zeiss Illuminator HXP 200C) and visualized through a 1X PlanNeoFluar Z 1.0x objective at 17x zoom.
 565 Images were captured at a resolution of 512 x 512 pixels (16-bit pixel depth) after 2 x 2 binning to
 566 increase sensitivity. Each recording consisted of uninterrupted acquisition over 10 minutes or 20
 567 minutes if injected with pharmacological agents.

568 **Catheterization of animals for *in vivo* imaging**

569 After induction of anesthesia and before installing the cranial window, a catheter was placed in
 570 the intraperitoneal (IP) space of neonatal mouse pups. A 24G needle was used to puncture the
 571 peritoneum and a small-diameter catheter (SAI Infusion Technologies, MIT-01) was placed. A drop
 572 of Vetbond secured the catheter to the pup's belly. Installation of cranial window proceeded as
 573 described above. Imaging sessions consisted of 5 minutes of baseline activity measurements, fol-
 574 lowed by a slow push of either 50µL of sham (5% mannitol solution) or MRS2500 solution (500µM
 575 in 5% mannitol solution). Imaging was continuous throughout and 20 minutes of activity total were
 576 collected. No discernable diminishment of activity was observed in sham animals.

577 **Image processing**

578 For wide field imaging, raw images were imported into the ImageJ environment and corrected for
 579 photobleaching by fitting a single exponential to the fluorescence decay and subtracting this com-
 580 ponent from the signal (Bleach Correct function, exponential fit). Images were then imported into
 581 MATLAB (Mathworks) and intensities were normalized as $\Delta F/F_0$ values, where $\Delta F = F - F_0$ and F_0 was
 582 defined as the fifth percentile value for each pixel. Ovoid regions of interest (ROIs) encompassing

583 the entire left and right inferior colliculi were drawn. Across all conditions, the size of the ROIs was
 584 invariant, however, due to small differences in the imaging field between animals, the ROIs were
 585 placed manually for each imaging session. Peaks in the signals were detected in MATLAB using
 586 the built-in peak detection function (*findpeaks*) using a fixed value threshold criterion; because
 587 fluorescence values were normalized, this threshold was fixed across conditions (2% $\Delta F/F_0$). Occa-
 588 sionally, large events in the cortex or superior colliculus would result in detectable fluorescence
 589 increases in the IC. These events broadly activated the entire surface of the IC and did not exhibit
 590 the same spatially-confined characteristics as events driven by the periphery. These events were
 591 not included in the analysis.

592 **Analysis of spatial distribution of activity in the IC**

593 As shown in *Figure 7D*, a rectangle of size 125 x 50 pixels was placed perpendicular to the tonotopic
 594 axis of the IC ($\pm 55^\circ$ rotation, respectively). The columns of the resulting matrix were averaged
 595 together to create a line scan (125 pixels x 1 pixel) for the entire time series. Peaks were detected
 596 using MATLAB's *imregionalmax* function with a constant threshold of 3% $\Delta F/F_0$ across all animals.
 597 Histograms of events along the tonotopic axis were generated by summing the number of events in
 598 25 μm bins. Lateral and medial designations were assigned by splitting the area evenly between the
 599 lateral edge and the location of defined single-band events in the medial portion of the IC. Events
 600 detected on the medial edge of single-band events, reflective of the bifurcation of this information,
 601 was not included in the medial/lateral analysis.

602 **Analysis of retinal wave activity in the superior colliculus**

603 ROIs (200 x 150 pixels) were placed over each lobe of the superior colliculus and downsampled by
 604 a factor of five. Signals were normalized as $\Delta F/F_0$ values, where $\Delta F = F - F_0$ and F_0 was defined as the
 605 fifth percentile value for each pixel. In order to eliminate periodic whole-sample increases in fluo-
 606 rescence, the mean intensity of all pixels was subtracted from each individual pixel. Following this,
 607 pixels were considered active if they exceeded the mean + 3 standard deviations. For each point in
 608 time, the number of active pixels was summed. Retinal waves were defined as prolonged periods
 609 (> 1 second), where more than 5 pixels were active simultaneously. Retinal wave durations were
 610 defined as the total continuous amount of time that more than 5 pixels were active. Frequencies
 611 and durations are similar to earlier reports (*Ackman et al., 2012*).

612 **Generation of the Tecta-Cre mouseline**

613 A crRNA (TAATGATGAATAATTCCATCC) targeted near exon 2 of the Tecta gene, tracrRNA, Cas9 re-
 614 combinase, and a donor plasmid containing an iCre-WPRE-polyA sequence (500 base pair homol-
 615 ogy arms) were injected into single-cell embryos that were then transferred to pseudopregnant
 616 recipient mothers. After birth, mouse pups were screened for insertion of the gene at the correct
 617 locus with two pairs of primers: one pair amplified DNA beginning 5' of the 5' homology arm and
 618 ending within the Cre sequence and the other amplified DNA within the polyA sequence and end-
 619 ing 3' of the 3' homology arm. These primers were then used to sequence the junctions. Of these,
 620 all mice used for experiments were derived from a single founder that was positive for both sets
 621 of primers and had 100% sequence validation. Mice were crossed to a TdTomato reporter line to
 622 examine cell-specific recombination (*Figure 4–Figure Supplement 1*).

623 **Quantification and statistical analysis**

624 All statistics were performed in the MATLAB (Mathworks) programming environment. All statistical
 625 details, including the exact value of n, what n represents, and which statistical test was performed,
 626 can be found in the figure legends. To achieve statistical power of 0.8 with of a 30% effect size with
 627 means and standard deviations similar to those observed in previous studies, power calculations
 628 indicated that 7 animals in each condition were necessary ($\mu_1 = 10$, $\mu_2 = 7$, $\sigma = 2$, sampling ratio = 1).
 629 While this number was used as a guide, power calculations were not explicitly performed before

630 each experiment; many experiments had much larger effect sizes and sample sizes were adjusted
 631 accordingly. For transparency, all individual measurements are included in the figures. Unless
 632 otherwise noted, data are presented as mean \pm standard error of the mean. All datasets were
 633 tested for Gaussian normality using the D'Agostino's K² test. For single comparisons, significance
 634 was defined as p <= 0.05. When multiple comparisons were made, the Bonferroni correction was
 635 used to adjust p-values accordingly to lower the probability of type I errors. For multiple condition
 636 datasets, one-way ANOVAs were used, followed by Tukey's multiple comparison tests.

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652 Declaration of Interests

653 The authors declare no competing financial interests.

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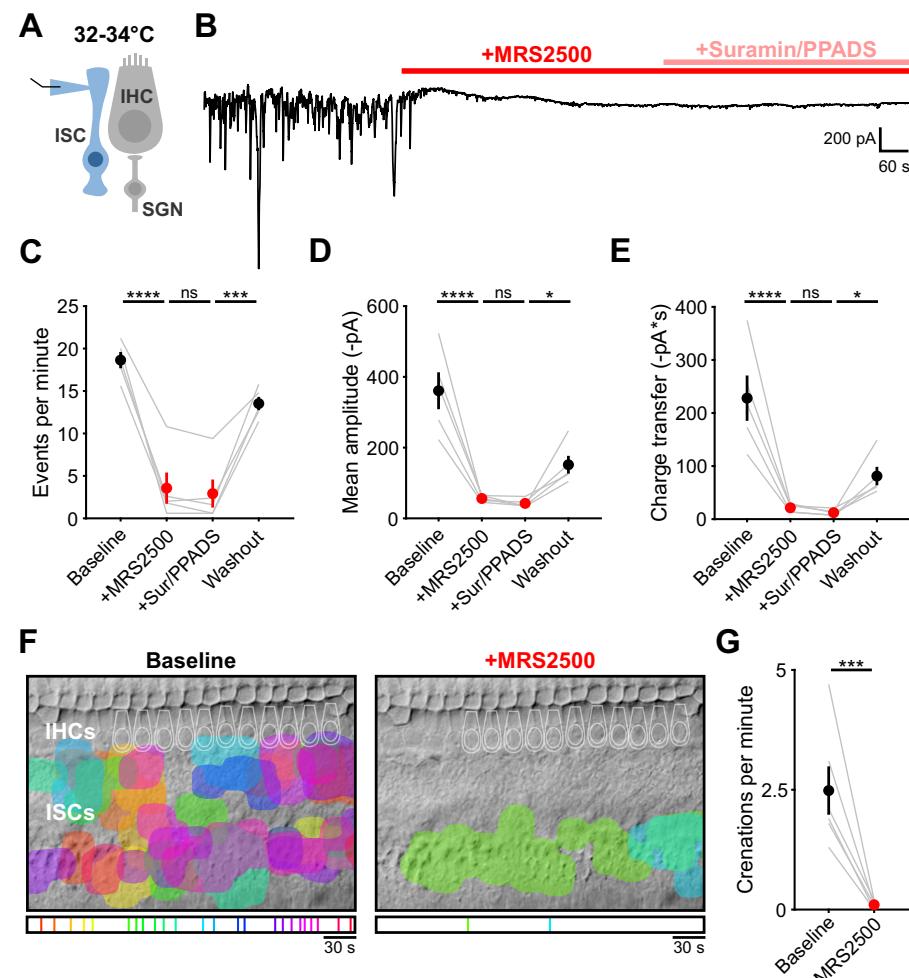


Figure 3-Figure supplement 1. P2RY1 inhibition abolishes spontaneous inward currents near physiological temperature. **(A)** Schematic of whole-cell recording configuration from ISCs. **(B)** Spontaneous inward currents recorded from an ISC before and after application of MRS2500 (1 μ M) and subsequent broad spectrum purinergic antagonists suramin (10 μ M) and PPADS (50 μ M). Recordings were performed near physiological temperature (32–34°C). **(C)** Plot of event frequency. Each window measured was 5 minutes in length, washout was taken 20 minutes after superfusion of aCSF. n = 5 ISCs from 5 cochleae (one-way ANOVA; ****p<5e-5, ***p<0.0005, ns, not significant). **(D)** Plot of event amplitude. n = 5 ISCs from 5 cochleae (one-way ANOVA; ****p<5e-5, *p<0.05, ns, not significant). **(E)** Plot of average integral (charge transfer). n = 5 ISCs from 5 cochleae (one-way ANOVA; ****p<5e-5, *p<0.05, ns, not significant). **(F)** Intrinsic optical imaging performed before and after application of the P2Y1 antagonist, MRS2500 (1 μ M). Detected crenations are outlined in colors based on time of occurrence as indicated by timeline below image. Imaging was performed near physiological temperature (32–34°C). **(G)** Plot of crenation frequency before and after MRS2500 application. n = 6 cochleae (two-tailed paired Student's t test; **p<0.005).

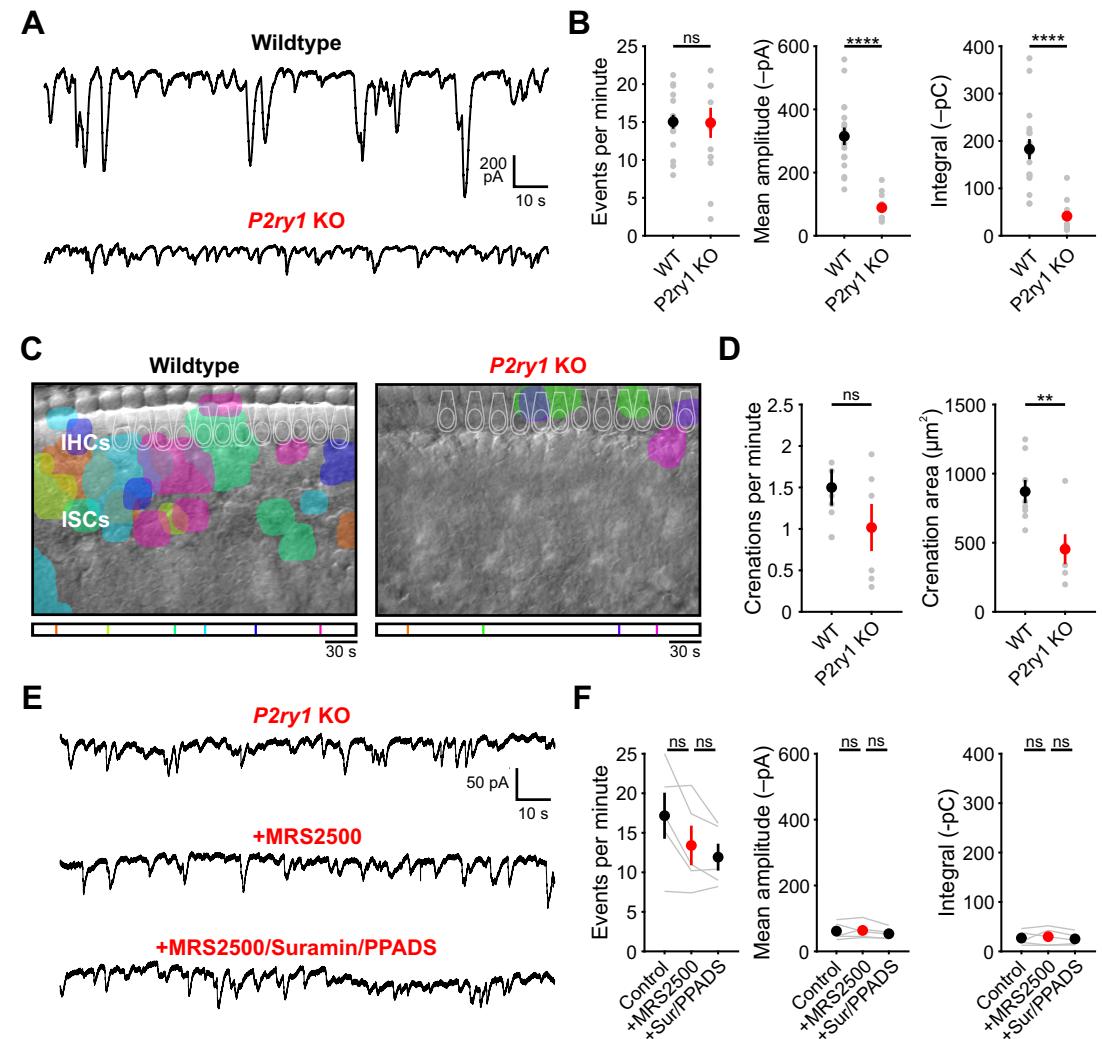


Figure 3-Figure supplement 2. Spontaneous inward currents and crenations are dramatically reduced in *P2ry1* KO mice. **(A)** Spontaneous inward currents recorded from ISCs in wildtype and *P2ry1* KO mice. Recordings were performed near physiological temperature (32–34°C). **(B)** Plots of event frequency, amplitude, and integral (charge transfer). n = 17 wildtype and 14 *P2ry1* KO ISCs (two-tailed Student's t-test with Bonferroni correction; ****p<0.0005, ns, not significant). **(C)** Intrinsic optical imaging performed in wildtype and *P2ry1* KO mice. Detected crenations are outlined in colors based on time of occurrence as indicated by the timeline below image. Imaging was performed at room temperature. **(D)** Plots of crenation frequency and area in wildtype and *P2ry1* KO mice. n = 8 wildtype cochleae and 6 *P2ry1* KO cochleae (two-tailed paired Student's t test with Bonferroni correction; **p<0.005, ns, not significant). **(E)** Spontaneous inward currents recorded from an inner supporting cell in *P2ry1* KO mice before and during application of MRS2500 (1μM) and subsequent broad spectrum purinergic antagonists suramin (100μM) and PPADS (50μM). **(F)** Plots of event frequency, amplitude, and charge transfer. n = 5 *P2ry1* KO ISCs (one-way ANOVA; ns, not significant).

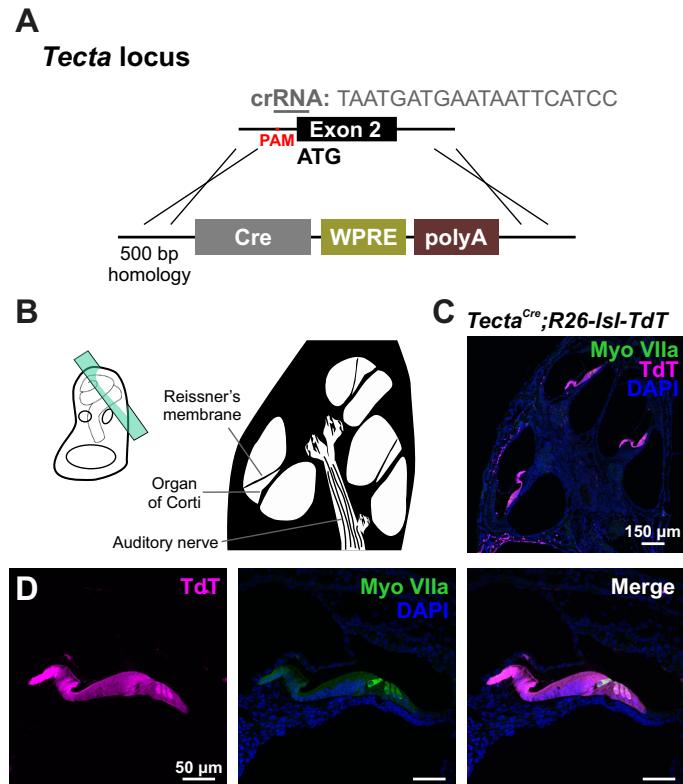


Figure 4–Figure supplement 1. Crispr-Cas9 mediated generation of the *Tecta*-Cre mouse line. **(A)** Targeting strategy for introducing an *iCre* coding sequence into the endogenous *Tecta* locus. Note: start ATG is located in exon 2. **(B)** Schematic of temporal bone with sectioning orientation indicated with green plane. **(C)** TdT reporter expression observed along the entire length of a P7 cochlea. Expression was absent in stria vascularis and very sparse in apical SGNs. **(D)** TdT reporter expression observed in nearly all cells within the sensory epithelium, including hair cells (MyoVIIa, green).

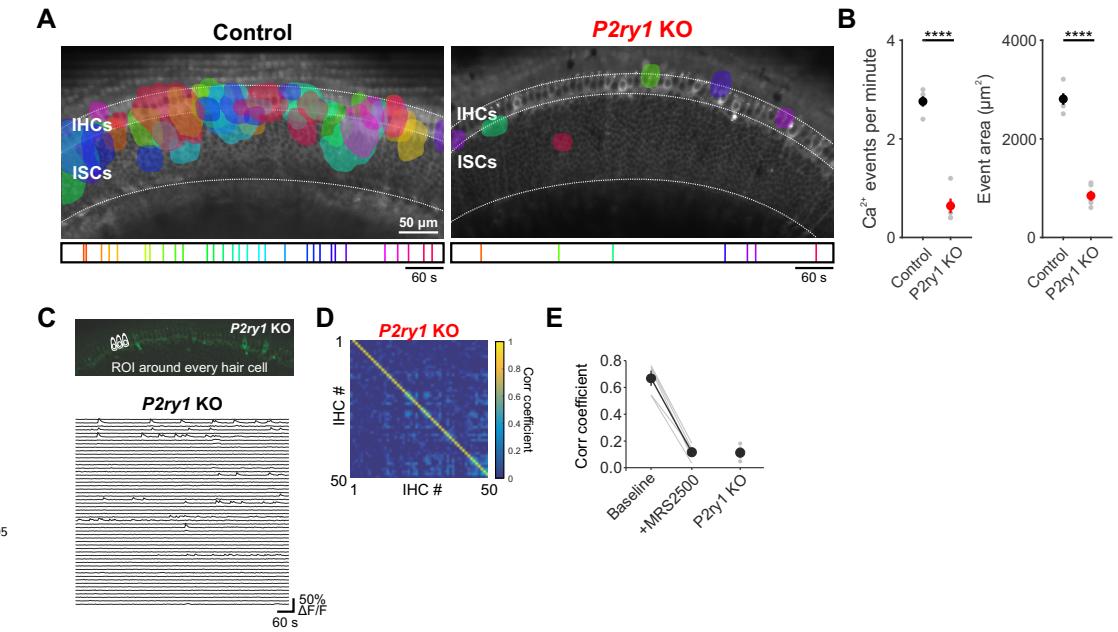


Figure 5-Figure supplement 1. *P2ry1* KO mice exhibit reduced Ca^{2+} transients in ISCs. **(A)** Maps showing maximum area of spontaneous Ca^{2+} transients in control (*Pax2-Cre;R26-*Isl-GCaMP3**) and *P2ry1* KO (*Pax2-Cre;R26-*Isl-GCaMP3*; P2ry1^{-/-}*) mice. Ca^{2+} transients in the ISC and IHC regions are color-coded based on time of occurrence as indicated in timeline below image. Imaging was performed at room temperature. **(B)** Plots of Ca^{2+} event frequency and area in control and *P2ry1* KO mice. $n = 5$ control and 5 *P2ry1* KO mice (two-tailed Student's t test with Bonferroni correction, **** $p < 0.05$). **(C)** Exemplar images of IHC Ca^{2+} transients. ROIs were drawn around every IHC for subsequent analysis (bottom). **(D)** Correlation matrices generated by calculating the linear correlation coefficient for all IHC pairs in *P2ry1* KO mice. **(E)** Plot of average correlation coefficient among the four nearest neighboring hair cells. Data from MRS2500 experiment (Figure 4) is reproduced here for comparison. $n = 5$ cochleae (two-tailed paired Student's t test with Bonferroni correction; *** $p < 0.0005$).

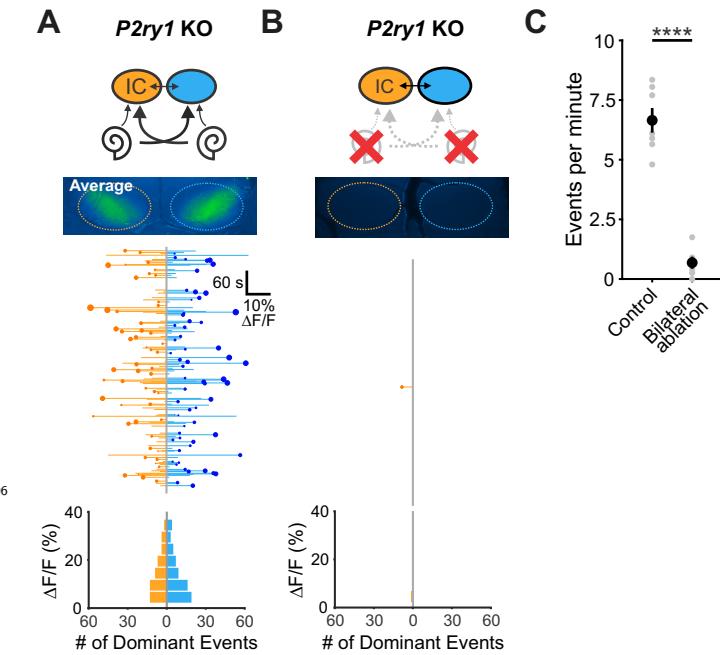


Figure 7-Figure supplement 1. Spontaneous activity in *P2ry1* KO mice originates in the cochlea. **(A)** Top: Diagram illustrating flow of information through the auditory system and average intensity image over the 10 min imaging session. Middle: Activity over time in left and right IC in an individual where each line indicates the fluorescence intensity of each detected event; the circle indicates the dominant lobe, and the size of the circle indicated the difference in fluorescence. Bottom: Histograms showing the frequency of dominant events of a given amplitude. **(B)** Similar to A, but with bilateral ablation of the cochleae. **(C)** Plot of IC event frequency in control (*P2ry1* KO) and bilaterally ablated (*P2ry1* KO) mice. $n = 7$ control and $n = 5$ bilaterally ablated *P2ry1* KO mice (two-tailed Student's t test; **** $p < 5e-5$).