Excess Extracellular K\(^+\) Causes Inner Hair Cell Ribbon Synapse Degeneration

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Excess extracellular K$^+$ causes inner hair cell ribbon synapse degeneration

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Inner hair cell (IHC) ribbon synapses are the first synapse in the auditory system and can be degenerated by noise and aging, thereby leading to hidden hearing loss (HHL) and other hearing disorders. However, the mechanism underlying this cochlear synaptopathy remains unclear. Here, we report that elevation of extracellular K$^+$, which is a consequence of noise exposure, could cause IHC ribbon synapse degeneration and swelling. Like intensity dependence in noise-induced cochlear synaptopathy, the K$^+$-induced degeneration was dose-dependent, and could be attenuated by BK channel blockers. However, application of glutamate receptor (GluR) agonists caused ribbon swelling but not degeneration. In addition, consistent with synaptopathy in HHL, both K$^+$ and noise exposure only caused IHC but not outer hair cell ribbon synapse degeneration. These data reveal that K$^+$ excitotoxicity can degenerate IHC ribbon synapses in HHL, and suggest that BK channel may be a potential target for prevention and treatment of HHL.
Noise is a common factor for hearing loss. It has been found that exposure to moderate levels of noise, even short-term, can cause extensive degeneration of ribbon synapses between inner hair cells (IHCs) and auditory nerves without hair cell loss. This cochlear synaptopathy can cause hidden hearing loss (HHL), which presents with a normal audiogram with difficulty in hearing speech in noisy environments. The cochlear IHC synapse degeneration also could impair hearing function in many aspects, such as neural adaptation, sound location, and temporal and speech processing. Such complaints account for ~10% of patients who present to otolaryngology clinics. In addition, the ribbon synapse degeneration is observed at the early stage of age-related hearing loss and may accelerate its progression. Glutamate is a major neurotransmitter of IHC ribbon synapses. It has been hypothesized that noise exposure may increase glutamate release, thereby leading to glutamate excitotoxicity which then causes hair cell death and synapse degeneration. However, the underlying mechanism for the cochlear synapse degeneration remains unclear.

The ribbon synapses between IHCs and auditory nerves are composed of presynaptic ribbons in IHCs and postsynaptic glutamate receptors (GluR) at endings of auditory nerves. Glutamate is the major neurotransmitter of IHC ribbon synapses. It has been found that presynaptic and postsynaptic components remained intact, despite the substantial terminal swelling following perfusion of GluR agonists. In a recent study, it demonstrated that noise could still induce ribbon synapse swelling in mice lacking vesicular glutamate transporter-3 with deficiency in release of glutamate. These data suggest that other mechanisms then glutamate excitotoxicity have an important role in the ribbon synapse degeneration.

On the other hand, noise-induced hair cell and auditory nerve excessive-excitation increases K⁺ efflux from hair cells and nerve fibers, which can elevate extracellular (perilymphatic) K⁺ concentration causing K⁺ excitotoxicity. Noise can also impair the reticular lamina integrity breaking the barrier between endolymph and perilymph, thereby leading to K⁺-rich endolymph (~150 mM) entering into the low-K⁺ perilymph (~5 mM K⁺) causing K⁺ toxicity. In the brain, it has been found that elevation of extracellular K⁺ resulting from cell death in the stroke area can cause secondary neuron degeneration in the neighboring non-stroke area. In this study, we tested whether high extracellular K⁺ could cause cochlear synapse degeneration. We found that elevation of extracellular K⁺ concentration could cause IHC ribbon synapse degeneration and swelling in a dose-dependent manner. Blockage of K⁺ channels attenuated this degeneration. However, application of GluR agonists did not cause IHC ribbon synapse degeneration, even though they caused ribbon swelling. These data suggest that K⁺ excitotoxicity may play a major role in the IHC ribbon synapse degeneration, thereby shedding light on the prevention and treatment of this common type of hearing loss.

Results

Degeneration of IHC ribbon synapses by elevation of extracellular K⁺. To test whether high extracellular K⁺ could cause IHC ribbon synapse degeneration, we incubated one cochlea in the high-K⁺ extracellular solution which contained 50 mM K⁺ for 2 h, as the same time as noise exposure to induce HHL. Another cochlea of the same mouse was simultaneously incubated in normal extracellular solution (NES) as control. Figure 1 shows that the IHC ribbon synapses were significantly reduced after challenge with 50 mM K⁺. In comparison with those in the NES group, the presynaptic ribbon (labeling puncta for CtBP2), postsynaptic GluR, and synapse (overlapped labeling) per IHC in the high-K⁺ group were reduced to 62.5 ± 3.20, 83.4 ± 5.94, and 60.8 ± 5.58% (P = 1.4E−05, 0.02, and 0.0008, paired t-test, two-tail), respectively (Fig. 1b). The reductions in ribbons and synapses were significantly larger than that in the GluR (P = 0.022 and 0.013, respectively, one-way ANOVA with a Bonferroni correction). However, there was no significant difference between ribbon reduction and synapse reduction (P = 1.00, one-way ANOVA). The percentages of synaptic ribbons and free ribbons in the high-K⁺ group and the NES group were almost the same (Supplementary Fig. 1a, b) and had no significant difference between two groups (P = 0.68, paired t-test, two-tail), indicating that high-K⁺ caused both synaptic ribbon and free ribbon degeneration similarly. Thus, in the following analyses, we would not distinguish synaptic ribbon and free ribbon degeneration and analyze the total ribbon degeneration. Figure 1c shows that the total ribbons in the apical, middle, and basal cochlear turn in the high-K⁺ group were significantly reduced to 72.1 ± 5.80%, 56.8 ± 3.73%, and 57.2 ± 7.65%, respectively, in comparison with those in the control group with NES incubation (P = 0.006, 5.4E−06, and 0.004, paired t-test, two-tail). The reduction in the apical turn appeared less than that in the middle and basal cochlear turns. However, there was no significant difference among them (P = 0.13, one-way ANOVA). High-K⁺ also caused ribbon swelling (Fig. 1d). In comparison with that in the control group with NES incubation, the ribbon volume in the high-K⁺ group was significantly increased 1.80 ± 0.22 folds (P = 0.003, paired t-test, two-tail).

We also tested the effect of the incubation procedure on the ribbon degeneration (Fig. 1e, f). One cochlea was incubated in the NES for 2 h and another cochlea in the same mouse was immediately fixed without incubation after isolation. The ribbon numbers in the incubation group and non-incubation group were 12.3 ± 1.01/IHC and 12.3 ± 0.96/IHC, respectively (Fig. 1e). There was no significant difference between them (P = 0.98, paired t-test, two-tail). The ribbon volume after incubation had a small increase and was increased 1.32-folds, i.e., increase of 32% (Fig. 1f, P = 0.002, paired t-test, two-tail). However, in comparison with the increase of 2.371-folds (i.e., increase of 137.1%, re; non-incubation group) in the high-K⁺ group (Supplementary Fig. 3b), the volume increase caused by the incubation procedure was significantly small (P = 0.02, one-way ANOVA with a Bonferroni correction).

K⁺-induced ribbon degeneration is dose-dependent (Fig. 2). As extracellular K⁺ concentration increased, the ribbon degeneration was increased. When the extracellular K⁺-concentration was greater than 30 mM, the ribbon degeneration reached a stable stage level. The median concentration of K⁺ was at 24.7 mM.

Attenuation of IHC ribbon synapse degeneration by K⁺ channel blockers. We further tested whether K⁺ channel blockers could attenuate this high-K⁺ induced IHC ribbon synapse degeneration. Since the Ca⁺⁺-dependent BK channel is a prevalent K⁺ channel at IHCs, the specific BK channel blocker iberiotoxin (IBT) was used (Fig. 3a, b). We incubated one cochlea in 50 mM K⁺ extracellular solution with 0.1 μM IBT (K⁺ + IBT group) and another cochlea of the same mouse in the 50 mM K⁺ extracellular solution (high-K⁺ group) as control. In comparison with those in the high-K⁺ control group, the survival of ribbons, GluRs, and synapses in the K⁺ + IBT group were increased by 151.5 ± 8.36%, 128.9 ± 7.73%, and 153.4 ± 14.0%.
$P = 0.0035, 0.0201, \text{ and } 0.0189,$ paired $t$-test, two-tail), respectively (Fig. 3a). In the apical, middle, and basal turn, the survival of ribbons was increased by $154.3 \pm 9.89\%$, $151.7 \pm 13.5\%$, and $165.5 \pm 14.0\%$ ($P = 0.0029, 0.0093, \text{ and } 0.0094,$ paired $t$-test, two-tail), respectively (Fig. 3b). If compared with those in the NES control group (Fig. 1c), the ribbon survival in the apical, middle, and basal turns in the $K^+_{IBT}$ group was increased from $72.1 \pm 5.80\%$, $56.8 \pm 3.73\%$, and $57.2 \pm 7.65\%$ (Fig. 1c) to $78.8 \pm 3.15\%$, $78.0 \pm 2.12\%$, and $81.6 \pm 11.8\%$, respectively, for 50 mM $K^+$ challenge. Thus, IBT could significantly increase IHC ribbon synapse survival for high-$K^+$ challenge.

Since IHCs also have other $K^+$ channels,$^{27,28}$ we further used a broad $K^+$ channel blocker tetraethylammonium (TEA). As the same as IBT, one cochlea was incubated with 50 mM $K^+$ extracellular solution with 20 mM TEA ($K^+_{-TEA}$ group), and another cochlea of the same mouse was incubated in 50 mM $K^+$ extracellular solution (high-$K^+$ group). The ribbon numbers in the different turns were normalized to that in the corresponding turn in the control cochlea of the same mouse. Figure 3c shows that 82.2 ± 1.85% of IHC ribbons in the $K^+_{-TEA}$ group was reserved for 50 mM $K^+$ challenge ($P = 0.01$, paired $t$-test, two-tail). In comparison with that (79.5 ± 6.47%) in the $K^+_{IBT}$ group, there was no significant difference between them ($P = 0.70$, $t$-test, two-tail). However, in comparison with that in the NES control group in Fig. 1, the ribbon volumes in the $K^+_{IBT}$ group and the $K^+_{-TEA}$ group were still increased 1.77 ± 0.24 folds and 1.80 ± 0.15 folds (Fig. 3d), respectively, and had no significant differences from those in the high-$K^+$ control groups ($P = 0.88$ and 0.95, respectively, paired $t$-test, two-tail). Thus, both IBT and TEA had no effect on the ribbon swelling.

**Fig. 1 High-$K^+$ induced IHC ribbon synapse degeneration.**

- **a** Immunofluorescent staining for ribbon synapses at the IHC area in the apical, middle, and basal turns after incubation. Two cochleas of a mouse were incubated separately in the high-$K^+$ (50 mM) extracellular solution and in the normal extracellular solution (NES) for 2 h. Presynaptic ribbons are labeled by CtBP2 (red) and postsynaptic GluRs are labeled by green. Scale bar: 10 µm.

- **b** Quantitative analyses of high-$K^+$ induced ribbon synapse degeneration at IHCs. Presynaptic ribbons, postsynaptic GluRs, and synapses per IHC pooled from all cochlear turns were averaged and normalized to those in the control cochlea of the same mouse with NES incubation. Dashed lines connected two same symbols represent data collected from two cochleas of the same mouse. $N$ is the animal number.

- **c** $K^+$-induced ribbon degeneration in the cochlear apical, middle, and basal turn. The ribbon numbers in the different turns were normalized to those in the corresponding turn in the control cochlea of the same mouse.

- **d** High-$K^+$ induced ribbon swelling. The volume of labeling puncta for CtBP2 were measured. The data pooled from all cochlear turns were averaged and normalized to that in the control cochlea of the same mouse. Total numbers of measured ribbons are presented within the bars. $N$ is the animal number.

- **e, f** Incubation procedure has no effect on ribbon degeneration. One cochlea was immediately fixed after isolation and stained for CtBP2 and another cochlea of the same mouse was incubated in NES for 2 h and then fixed for staining. Dashed lines connected two same symbols represent data collected from two cochleas of the same mouse. Data were pooled from all cochlear turns. Numbers in the bars represent the total numbers of accounted IHCs and measured ribbons. $N$ is the animal number. $* P < 0.05$, $** P < 0.01$, paired $t$-test, two-tail for all panels.
Attenuation of Ca\textsuperscript{++} channel blockers on IHC ribbon synapse degeneration and swelling. Since BK channels could be Ca\textsuperscript{++}-dependent, we further tested whether blockage of Ca\textsuperscript{++} channels could attenuate the high-K\textsuperscript{+} induced IHC ribbon synapse degeneration. Co\textsuperscript{++} is a broad Ca\textsuperscript{++} channel blocker. As the same as application of IBT and TEA, we added 2 mM Co\textsuperscript{++} into the high-K\textsuperscript{+} (50 mM) incubation solution for one cochlea incubation (K\textsuperscript{++}Co\textsuperscript{++} group) and another cochlea of the same mouse was incubated with 50 mM K\textsuperscript{+} extracellular solution (high-K\textsuperscript{+} group). The ribbon numbers in both groups were normalized to that in the NES control group in Fig. 1. Figure 4a showed that the ribbon survival was significantly increased from 50.8 ± 7.00% in the high-K\textsuperscript{+} group to 72.7 ± 7.07% in the K\textsuperscript{++}Co\textsuperscript{++} group (P = 0.006, paired t-test, two-tail). The ribbon swelling (Fig. 4b) was also significantly reduced from 1.80 ± 0.16 folds in the high-K\textsuperscript{+} group to 1.29 ± 0.14 folds in the K\textsuperscript{++}Co\textsuperscript{++} group (P = 0.005, paired t-test, two-tail). Thus, the Ca\textsuperscript{++} channel blocker Co\textsuperscript{++} could attenuate both ribbon degeneration and swelling.

We also used the extracellular ionic blocking solution (EIBS), which contains 20 mM TEA, 2 mM Co\textsuperscript{++}, and 20 mM Cs\textsuperscript{+}, to block both K\textsuperscript{+} and Ca\textsuperscript{++} channels extensively. First, we examined the attenuating effect of EIBS in the paired experiment of high-K\textsuperscript{+} vs. high-K\textsuperscript{+} with EIBS. One cochlea was incubated in the high-K\textsuperscript{+} (50 mM) extracellular solution (high-K\textsuperscript{+} group) and another cochlea of the same mouse was incubated in the NES (K\textsuperscript{+} = 5.37 mM) as control. The data pooled from all cochlear turns were averaged and normalized to that in the control cochlea of the same mouse. N is the mouse number at each data point. A smooth solid line represents data fitting to a Hill’s function \( y = y_0 + a \cdot C^n/(EC_{50}n + C^n) \), where median concentration EC\textsubscript{50} = 24.7 mM.
Fig. 4 Attenuation of IHC ribbon degeneration and swelling by Ca++ channel blocker. a, b Attenuation of the Ca++ channel blocker Co++ on ribbon degeneration and swelling induced by high-K+ challenge. Two cochleas of the same mouse were incubated separately in the 50 mM K+ extracellular solution with 2 mM Co++ (K+++Co++ group) and 50 mM K+ extracellular solution (high-K+ group). Ribbon numbers and volumes pooled from all cochleas of each group were separately averaged and normalized to that in the NES control group in Fig. 1. Dashed lines connected two same symbols represent data collected from two cochleas of the same mouse. N is the animal number. Number in each bar represents IHC or ribbon numbers. Co++ significantly attenuates both ribbon degeneration and swelling induced by high-K+.

c, d Attenuation of high-K+ induced ribbon degeneration and swelling by extracellular ionic blocking solution (EIBS), which contains 20 mM TEA, 20 mM Cs+, and 2 mM Co++ and can block both Ca++ and K+ channels extensively in the hair cells. One cochlea in a mouse was incubated in the high-K+ (50 mM) extracellular solution (high-K+ group) and another cochlea was incubated in the 50 mM K+ with EIBS (K+++EIBS group) for 2 h. Ribbon numbers and volumes in both the high-K+ group and the K+++EIBS group were separately normalized to those in the NES control group in Fig. 1. **P < 0.01, paired t-test, two-tail.

Another cochlea was incubated with 50 mM K+ plus EIBS (K++ +EIBS group). The ribbon numbers in both groups were normalized to that in the NES control group in Fig. 1. Figure 4c shows that 95.5 ± 3.0% of IHC ribbons in the K+++EIBS group were reserved for 50 mM K+ challenge (P = 0.002, paired t-test, two-tail). Figure 4d also shows that the ribbon volume in the high-K+++EIBS group was only increased 1.24 ± 0.17 folds. In comparison with increase of 1.80 ± 0.20 folds in the ribbon volume in the high-K+ group, the ribbon swelling in the high-K+++EIBS group was reduced by 70.0% (P = 0.0007, paired t-test, two-tail). We also examined the attenuating effect of EIBS in the paired experiment of NES vs. K+++EIBS, in which one cochlea was incubated with NES and another cochlea was incubated in EIBS with 50 mM K+ (Supplementary Fig. 2). In comparison with those in the control NES group, the ribbon number and volume in the K+++EIBS group were 98.9 ± 3.96% and 1.23 ± 0.21 folds, respectively (Supplementary Fig. 2). There were no significant differences in the ribbon number and volume between the NES and K+++EIBS groups (P = 0.73 and 0.32, respectively, paired t-test, two-tail). In both paired experiments, the ribbon survival in the K+++EIBS group was >95% and the ribbon volume was also only increased ~1.2-folds, similar to the volume increase (1.32-folds) caused by incubation procedure itself (Fig. 1f). Thus, blockage of both Ca++ and K+ channels could almost completely attenuate high-K+ induced IHC ribbon degeneration and swelling.

Figure 5 shows the comparison of attenuations of K+ and Ca++ channel blockers on the ribbon degeneration and swelling. All of K+ and Ca++ blocker TEA, IBT, Co++, and EIBS could significantly attenuate high-K+ induced ribbon degeneration (P < 0.001, one-way ANOVA with a Bonferroni correction, Fig. 5a). EIBS had a maximum of attenuation and could allow 95.5% of ribbons survival for high-K+ challenge, whereas Co++ had 72.7% of minimum attenuation. There was significant difference between them (P = 0.008, one-way ANOVA with a Bonferroni correction). However, there was no significant difference among attenuations of TEA, IBT, and Co++ in the ribbon degeneration (P = 0.55, one-way ANOVA). For ribbon swelling (Fig. 5b), there was no significant reduction after application of K+ channel blockers (P = 1.00, one-way ANOVA). However, there was significant reduction in the ribbon volumes after application of Ca++ channel blockers. The ribbon volumes in the K+++Co++ group and the K+++EIBS group were only increased to 1.29 ± 0.14 and 1.24 ± 0.17 folds, respectively. In comparison with ~1.8-folds of increase in the high-K+, K+ -IBT, and K+ -TEA groups, the ribbon swelling in the K+++Co++ group and K+++EIBS group...
GluR agonists cause IHC ribbon synapse swelling but not degeneration. To assess the effect of glutamate excitotoxicity on the IHC ribbon synapse degeneration, we investigated the effect of GluR agonists on IHC ribbon degeneration. In the same way, two cochleas in the same mouse were separately incubated with 0.2 mM glutamate and NES for 2 h. Figure 6a shows that in comparison with those in the control NES group, the numbers of ribbons, GluRs, and synapses in the glutamate incubation group were $107 \pm 6.98\%$, $104 \pm 6.04\%$, and $93.5 \pm 5.75\%$ ($P = 0.42$, $0.42$, and $0.30$, paired t-test, two-tail), respectively. Also, in comparison with those in the NES control group, the numbers of ribbons in the apical, middle, and basal turns in the glutamate group were $98.1 \pm 8.67\%$, $101 \pm 6.43\%$, and $110 \pm 10.3\%$, respectively (Fig. 6b).

There were no significant differences in ribbon numbers in the apical, middle, and basal turns between two groups ($P = 0.78$, $0.91$, and $0.45$, paired t-test, two-tail). However, glutamate could cause ribbon swelling (Fig. 6c, d). In comparison with that in the control group, the volume of ribbons in the glutamate group was significantly increased $2.08 \pm 0.15$ folds (Fig. 6c, $P = 0.0002$, paired t-test, two-tail). The swelling was not significantly different from that caused by the high-K$^+$ challenge ($P = 0.72$, one-way ANOVA, Supplementary Fig. 3b).

Application of GluR agonist Kainic acid (KA, 0.3 mM) or NMDA (0.3 mM) also did not cause ribbon degeneration (Fig. 7a, c). In comparison with those in each NES control group, the ribbon numbers in KA and NMDA groups were $97.4 \pm 8.56$ and $101.4 \pm 10.2\%$, respectively ($P = 0.70$ and 0.83, paired t-test, two-tail). However, like glutamate, KA, and NMDA caused ribbon swelling (Fig. 7b, d). In comparison with that in the NES control group, the ribbon volumes in the KA group and NMDA group were significantly increased $1.65 \pm 0.20$ and $1.75 \pm 0.24$ folds, respectively ($P = 0.01$ and 0.04, respectively, paired t-test, two-tail). Thus, GluR agonists glutamate, KA, and NMDA could cause IHC ribbon swelling but not degeneration.

However, a GluR (AMPA/Kainate receptor) antagonist cyan- quinidine (CNQX) could attenuate the K$^+$-induced ribbon degeneration but not swelling (Fig. 7e, f). After application of 0.1 mM CNQX with 50 mM K$^+$, the survival of ribbon numbers had a small increase from $50.8 \pm 6.55\%$ in the high-K$^+$ group to $62.5 \pm 9.05\%$ in the K$^+$+CNQX group ($P = 0.014$, paired t-test, two-tail). However, there was no significant difference in the ribbon volume between the high-K$^+$ group ($1.80 \pm 0.18$ folds) and the K$^+$+CNQX group ($1.60 \pm 0.09$ folds) ($P = 0.22$, paired t-test, two-tail).

IHC ribbon synapse degeneration after noise exposure. We further assessed IHC ribbon degeneration after noise exposure (Fig. 8). We first assessed mouse hearing functional changes for noise exposure by auditory brainstem response (ABR) recording. Before noise exposure, ABR thresholds were $22.0 \pm 0.5$, $22.3 \pm 1.08$, $14.5 \pm 1.38$, $19.5 \pm 0.82$, $25.3 \pm 0.58$, and $28.3 \pm 1.06$ dB SPL in the control group and $21.3 \pm 0.67$, $19.3 \pm 2.04$, $11.0 \pm 1.45$, $160.0 \pm 1.55$, $22.0 \pm 1.04$, and $27.0 \pm 2.41$ dB SPL in the noise exposure group for click, 8, 16, 24, 32, and 40 kHz tone stimulations, respectively (Fig. 8a). There were no significant differences in the pre-noise exposure ABR thresholds between two groups ($P > 0.05$, one-way ANOVA). After exposure to white noise (95–98 dB SPL) for 2 h, ABR thresholds measured at immediately stop of the exposure were $39.0 \pm 1.83$, $52.7 \pm 3.40$, $61.0 \pm 2.17$, $67.3 \pm 1.95$, $67.3 \pm 3.75$, and $67.5 \pm 4.35$ dB SPL in the control group and $21.3 \pm 0.67$, $19.3 \pm 2.04$, $11.0 \pm 1.45$, $160.0 \pm 1.55$, $22.0 \pm 1.04$, and $27.0 \pm 2.41$ dB SPL in the noise exposure group for click, 8, 16, 24, 32, and 40 kHz tone bursts, respectively (Fig. 8a). In comparison with those at pre-noise exposure, the ABR thresholds were significantly elevated by ~40 to 50 dB SPL ($P < 0.001$, one-way ANOVA with a Bonferroni correction). IHC ribbons in the control mice and noise-exposure mice were $13.9 \pm 0.34$ and $8.97 \pm 0.62$ per IHC, respectively (Fig. 8b, c), and were reduced by 64.5% in the noise-exposure mice ($P < 0.001$, one-way ANOVA with a Bonferroni correction). After noise exposure, ribbons also appeared swollen (Fig. 8d). The ribbon volumes in the control mice and the noise-exposed mice were $0.418 \pm 0.029$ and $0.647 \pm 0.085 \mu m^3$ ($P = 0.035$, one-way ANOVA with a Bonferroni correction), respectively; there was 1.55-folds increase in the noise exposure group.

No outer hair cell ribbon synapse degeneration for high-K$^+$ challenge and noise exposure. However, there were no significant reductions in outer hair cell (OHC) ribbons for high-K$^+$ challenges and noise exposure. Figure 9a–c show that there was...
no significant reduction in OHC ribbons after application of 50 mM K\(^+\). The numbers of OHC ribbons in the high-K\(^+\) (50 mM) group and the NES control group were 2.43 ± 0.04 and 2.40 ± 0.04 per OHC, respectively (Fig. 9c, \(P = 0.31\), one-way ANOVA). Also, there was no significant reduction in OHC ribbons in noise exposed mice (Fig. 9d–f). The ribbon numbers in the noise exposure and control mice were 2.47 ± 0.04 and 2.38 ± 0.05 per OHC, respectively (Fig. 9f, \(P = 0.88\), one-way ANOVA).

Discussion
In the present study, we found that elevation of extracellular K\(^+\) could cause IHC ribbon synapse degeneration and swelling (Fig. 1). The degeneration was dose-dependent and increased as K\(^+\) concentration was increased (Fig. 2). K\(^+\) channel blockers, especially BK channel blockers, could attenuate ribbon degeneration but not swelling, whereas the Ca\(^{++}\) channel blocker Co\(^{++}\) attenuated both ribbon degeneration and swelling (Figs. 3–5). However, GluR agonists only caused ribbon swelling but not degeneration (Figs. 6 and 7). A GluR (AMPA/Kainate receptor) antagonist CNQX could attenuate K\(^+\)-induced ribbon degeneration but not swelling (Fig. 7e, f). Finally, both high-K\(^+\) challenge and noise exposure caused only IHC ribbon degeneration but not OHC ribbon degeneration (Figs. 8 and 9). These data suggest that high extracellular K\(^+\) can degenerate IHC ribbon synapse degeneration in HHL.

As mentioned above, the ribbon synapse degeneration has been hypothesized to result from glutamate excitotoxicity; acoustic overstimulation (noise exposure) results in excessive neurotransmitter glutamate release, thereby leading to excitotoxicity and hair cell and ribbon synapse degeneration\(^{17}\). However, cochlear synaptopathy in HHL has only ribbon synapse degeneration without hair cell death. Previous experiments also demonstrated that the presynaptic and postsynaptic components remained intact after perfusion of GluR agonists\(^19,20\). In the present study, we found that application of GluR agonists caused ribbon swelling but not degeneration; there were no significant degenerations in presynaptic ribbons, postsynaptic GluRs, and synapses after application of GluR agonists (Figs. 6, 7 and Supplementary Fig. 3). In addition, noise still could cause IHC ribbon swelling and degeneration in mice lacking vesicular glutamate transporter-3 and glutamate release\(^18\). These results suggest that glutamate excitotoxicity may not be the main mechanism for IHC ribbon synapse degeneration.

When sensory cells and neurons are excited, intracellular K\(^+\) is released to the extracellular space to restore polarization. Excessive excitations of hair cells and neurons can increase K\(^+\) release and elevate extracellular K\(^+\), thereby leading to K\(^+\) excitotoxicity. In this study, we found that elevation of K\(^+\) caused IHC ribbon synapse degeneration (Fig. 1 and Supplementary Fig. 3). Blockage of K\(^+\) channels, in particular, BK channels attenuated this...
The same mouse was incubated in 50 mM K⁺ to induce hearing loss in mice, although it had a report that BK channel activity by genetic deletion could increase resistance to noise-induced cochlear synaptopathy. One cochlea was incubated in 50 mM K⁺ extracellular solution with 0.1 mM CNQX (K⁺+CNQX group) and another cochlea of the same mouse was incubated in 50 mM K⁺ extracellular solution (high-K⁺ group). Ribbon numbers and volumes in the high-K⁺ group and the K⁺+CNQX group were normalized to those in the NES control group in Fig. 1.

Fig. 7 Effects of GluR agonist and antagonist on IHC ribbon degeneration and swelling. a-d Application of 0.3 mM kainic acid (KA) or 0.3 mM NMDA causes ribbon swelling but not degeneration. The ribbon number and volume pooled from all cochlear turns were averaged and normalized to those in the control cochlea with NES incubation in the same mouse. N is the number of mice. e, f Effects of a GluR antagonist CNQX on K⁺+induced ribbon degeneration and swelling. One cochlea was incubated in 50 mM K⁺ extracellular solution with 0.1 mM CNQX (K⁺+CNQX group) and another cochlea of the same mouse was incubated in 50 mM K⁺ extracellular solution (high-K⁺ group). Ribbon numbers and volumes in the high-K⁺ group and the K⁺+CNQX group were normalized to those in the NES control group in Fig. 1. N is the number of mice. * P < 0.05, ** P < 0.01, and n.s. no significance (P > 0.05), paired t-test, two-tail for all panels.

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The BK channel is a predominant type of K⁺ channels in mature IHCs. There are two types of BK channels in the mature IHCs: one is IBT-sensitive but can be blocked by TEA and Ca²⁺. Both IBT and TEA could attenuate IHC ribbon degeneration (Figs. 3 and 5). The IBT-sensitive BK channel is Ca²⁺-dependent and can be activated by Ca²⁺++ flowing through L-type Ca²⁺++ channels (Cav1.3). In this study, application of Co²⁺++ attenuated high-K⁺-induced ribbon degeneration (Figs. 4 and 5). High-K⁺ could depolarize IHCs to open Ca²⁺++ channels. Co²⁺++ may block Ca²⁺++ channels and consequently Ca²⁺++-dependent BK channels in IHCs. Moreover, it was reported that deficiency of BK channel activity by genetic deletion could increase resistance to noise-induced hearing loss in mice, although it had a report that BKα gene deletion could increase noise-induced hearing loss in low frequency regions with a higher noise vulnerability of OHCs. Taken together, these data suggest that BK channels may play a critical role in the IHC ribbon synapse degeneration. These data also suggest that BK channels may be a promising target for prevention and treatment of ribbon synapse degeneration in HHL.

In the experiments, we found that the GluR (AMPA/Kainate receptor) antagonist CNQX could attenuate high-K⁺-induced ribbon degeneration in IHCs (Fig. 7e). IHCs have Ca²⁺++-permeable GluR4-assembled AMPA receptor expression. Thus, CNQX may block presynaptic GluR4 AMPA receptors and reduce Ca²⁺++ influx, thereby blocking Ca²⁺++-activated BK channels in IHCs to attenuate ribbon degeneration as Co²⁺++ did. Indeed, a recent study reported that blockage of Ca²⁺++-permeable AMPA receptors by IEM-1460 in vivo could attenuate noise-induced HHL and synaptopathy in mice. Noise exposure could increase extracellular K⁺ in the cochlea in vivo. Thus, blockage of Ca²⁺++-permeable AMPA receptors may consequently block Ca²⁺++-dependent BK channels as well in the IHCs to attenuate cochlear synaptopathy (Fig. 7f), thereby eventually attenuating noise-induced HHL.

The present study demonstrated that TEA and IBT only attenuated ribbon degeneration (Figs. 3 and 5), whereas Co²⁺++ could attenuate both ribbon degeneration and swelling (Figs. 4 and 5). These data suggest that ribbon degeneration and swelling may underlie different mechanisms. Previous studies demonstrated that Ca²⁺++ excitotoxicity plays an important role in ribbon swelling. In the IHC, Ca²⁺++ can influx through Cav1.3 channels. High-K⁺ depolarizes the IHC and can open Cav1.3 channels to influx Ca²⁺++ channels. Co²⁺++ could block Ca²⁺++ channels and eliminate such Ca²⁺++ influx, thereby attenuating ribbon swelling (Figs. 4 and 5). Ca²⁺++ can also influx into IHCs through GluRs. As mentioned above, Ca²⁺++-permeable GluR4 AMPA receptors are expressed at presynaptic IHCs. Thus, application of GluR agonists could induce Ca²⁺++ influx and ribbon swelling but not degeneration (Figs. 6 and 7). Noise still caused IHC ribbon swelling in mice lacking the vesicular glutamate transporter-3, both Ca²⁺++ channel-dependent and GluR-dependent pathways may work together in vivo. Currently, it is still unclear and under debating whether ribbon swelling is recoverable or eventually leads to degeneration. However, during two-hour incubation with GluR agonists in this study, we...
only found significant IHC ribbon swelling but not degeneration (Figs. 6, 7 and Supplementary Fig. 3). Therefore, these data suggest that Ca\(^{2+}\) excitotoxicity plays an important role in IHC ribbon swelling and may also have a role in the IHC ribbon degeneration in vivo.

IHC synapses in vivo are embedded in the low-K\(^+\) perilymph and K\(^+\)-level in the perilymph is normally as low (~5 mM). However, when the sensory cells and neurons are excited, they will release intracellular K\(^+\) to the perilymph increasing K\(^+\) concentration in the perilymph. The K\(^+\) concentration in the local extracellular space around hair cell’s synapse area even could be higher. Moreover, noise, aging, or other factors could impair the integrity of the reticular lamina and the barrier between the endolymph and perilymph and allow K\(^+\)-rich endolymph (K\(^+\) = ~150 mM) entering the perilymph\(^{21,22}\). All of these changes could lead to elevation of extracellular K\(^+\) concentration, thus causing K\(^+\) excitotoxicity and cell depolarization. In the brain, it is well-known that neuron damage by stroke can cause intracellular K\(^+\) release into the extracellular space leading to K\(^+\) excitotoxicity, which can cause the secondary cell death in the neighboring non-stroke area\(^{23,24}\). In this study, we found that high-extracellular K\(^+\) could cause IHC ribbon synapse degeneration (Figs. 1–5). Like intensity dependence of ribbon synapse degeneration in noise exposure\(^{41}\), the degeneration was dose-dependent (Fig. 2).

Blockage of K\(^+\) channels attenuated ribbon degeneration but not swelling (Figs. 3–5). In addition, both K\(^+\) and noise caused IHC ribbon degeneration but not OHC ribbon degeneration (Fig. 9). Therefore, these data suggest that K\(^+\) excitotoxicity may play an important role in the noise-induced cochlear synapse degeneration and HHL.

This study may also provide important information for mechanisms underlying other cochlear and hearing disorders, such as Meniere’s disease. Its symptoms have been considered to result from K\(^+\) intoxicating the hair cells and nerve fibers in the perilymph\(^{42,43}\). It was reported that there was a significant decrease in the number of afferent nerve endings and afferent synapses at the base of IHCs in Meniere’s disease\(^{44}\), consistent with our finding in this study that high-K\(^+\) could cause IHC ribbon synapse degeneration (Figs. 1–5). Our study, therefore, may also provide important information for understanding mechanisms of Meniere’s disease.

### Materials and methods

**Animal and solution preparations.** Both genders of adult CBA/CaJ mice (8–12 weeks old, Stock No: 000654, The Jackson Lab) were used. Mice were housed in a quiet individual room in basement with regular 12/12 light/dark cycle. The background noise level in the mouse room at mouse hearing range (4–70 kHz) is <30 dB SPL. All procedures and experiments followed in the use of animals were approved by the University of Kentucky’s Animal Care & Use Committee (UK:
Cochlear preparation and high-K⁺ challenge. The mouse cochlea was freshly isolated and the temporal bone was removed. The otic capsule was opened. The bone that encloses the cochlea was gently crushed and removed. Then, the tectorial membrane and stria vascularis were carefully torn off by forceps. The basilar membrane with the organ of Corti (auditory sensory epithelium) was picked away by a sharpened needle and collected for staining. Starting from the cochlear apical region, the area at 25, 50, and 75% was considered as the apical, middle, and basal turn, respectively.

Immunofluorescent staining and confocal microscopy. Both inner ears in the same mouse were stained in parallel to minimize variations of the performance. The isolated cochlear sensory epithelia were incubated in a blocking solution (10% goat serum and 1% BSA of PBS) with 0.1% Triton X-100 for 30 min. Then, the epithelia were incubated with primary antibodies for CtBP2 (1:200, mouse anti-CtBP2 IgG1, #612044, BD Bioscience) and GluR (1:500, mouse anti-GluR2 IgG2a, #MAB397, Millipore Corp.) in the blocking solution at 4 °C overnight. In some of the early experiments, only anti-CtBP2 antibody was used. After washout, the epithelia were incubated with corresponding secondary Alexa Fluor antibodies (1:500, goat anti-Alexa Fluor 568, A-21124, or with goat anti-Alexa Fluor 488, A-21131, Thermo Fisher Sci) at room temperature (23 °C) for 2 h. Before washout, 4′,6-diamidino-2-phenylindole (DAPI, 50 µg/ml, D1306; Thermo Fisher Sci) was added to visualize the cell nuclei. After incubation for 2 min, the sensory epithelia were washed by PBS three times and whole-mounted on the glass slide. The stained epithelia were observed under a Nikon A1R confocal microscope system. Images were taken along the cochlear sensory epithelium from the apical to the basal turn.
turn to the basal turn. At each turn, at least 2–3 view-fields were imaged. At each field, serial sections were scanned along the Z-axis from the bottom to apical surface of the epithelium with a 0.25 μm step by use of a Nikon 100× (NA: 1.45) Plan Apo oil objective at 0.25 μm/pixel resolution.

Quantitative analysis and image presentation. The maximum intensity projection was generated from z-stack images at each image field. The numbers of ribbons (puncta of CtBP2 labeling), GluR labeling, and synapses (contacted or overlapped CtBP2 and GluR labeling) in the IHC region were separately accounted by NIS Elements AR Analysis software (Nikon). The IHC numbers in the field were also accounted. The number per IHC was calculated and averaged from all imaged fields in each cochlear turn or the whole cochlea. Then, they were normalized to those in the control ear of the same mouse. Finally, the numbers among different animals within a given experiment were averaged and compared. In some cases, the number per OHC was also calculated.

The ribbon volumes were measured from the 3D-image created from z-stack sections by Imaris software (Bitplane Inc., MA, USA). The vesicle volume measure function in the Imaris software was used with 0.15 μm smoothing. The volume measurement was performed by defining a minimum threshold above local background and a minimum acceptable Guassian distribution of voxel intensity to minimize false-positive noise. Similar parameters were used in the measurements among different mice. The volumes of IHC ribbons were measured from at least three different imaging fields in each cochlea and averaged. The means was normalized to that of the control ear of the same animal and further averaged among different animals within a given experiment.

Noise exposure. Littermate mice (8–10 weeks old) in one cage were randomly separated to the noise exposure group and the control group to ensure age and sex-matched control. Mice were awake in a small cage under loud-speakers in a sound-proof chamber and exposed to white-noise (95–98 dB SPL) for 2 h, one time. Noise was amplified by a power amplifier (CSA 2402, JBL Commercial, UT, USA). Sound pressure level and spectrum of noise in the cage were measured and verified prior to placement of mice.

Auditory brainstem response recording. Auditory brainstem response (ABR) was recorded by use of a Tucker-Davis R3 workstation with E3-1 high frequency speaker (Tucker-Davis Tech., Alachua, FL). For ABR recording, mice were anesthetized with a mixture of Ketamine and Xylazine (1 ml Ketamine+0.55 ml Xylazine+8.5 mL saline, 0.1 mL/10 g body weight, i.p.). Body temperature was maintained at 37–38 °C by placing anesthetized mice on an isothermal pad (Deltech, model 394p, Braintree Scientific Inc., Massachusetts). ABR was recorded by simulation with clicks in alternative polarity and tone bursts (4–40 kHz) from 80 to 10 dB SPL in a 5 dB step in a double-walled sound isolation room. The signal was amplified (50,000×), filtered (300–3000 Hz), and averaged by 500 times. The ABR threshold was determined by the lowest level at which an ABR can be recognized. ABR was recorded at 1–2 days before noise exposure as reference and immediately after stop of noise exposure to assess the threshold shift by noise exposure.

Statistics and reproducibility. Data were plotted by SigmaPlot (version 13, Systat Software) and statistically analyzed by Excel (Microsoft Office 360) or SPSS (version 25, SPSS Inc.; Chicago, IL). Error bars represent SEM. Parametric and nonparametric data comparisons were performed using one-way ANOVA or Student t-tests after assessment of normality and variance. The paired, two-tailed Student’s t-test was used for comparison of measures within the same group. The threshold for significance was P = 0.05. Bonferroni post hoc test was used in ANOVA.

For in vitro experiments, two inner ears in one mouse were used at each time. Each in vitro experiment was repeated at least four times to verify the reproducibility. Major experiments for high-K+ challenge and glutamate treatment were repeated for more than ten times with different experimenters (see Supplementary Fig. 3). For in vivo noise exposure experiment, only one or two mice were exposed at each time. Totally, five mice were exposed to noise and three times were repeated.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

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**Author contributions**
H.B.Z. conceived the general framework of this study. Y.Z., L.M.L., and H.B.Z. performed the experiments. H.B.Z., Y.Z., and L.M.L. analyzed data. H.B.Z. wrote the paper. All authors reviewed the manuscript and provided the input.

**Competing interests**
The authors declare no competing interests.

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