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14. ABSTRACT

Noise-induced synaptopathy is the result of excitotoxic trauma to cochlear synapses due to glutamate released from the hair cells. Excitotoxic trauma damages the postsynaptic cell by causing entry of Ca2+ ions. We have identified the route of Ca2+ entry as via Ca2+-permeable AMPA-type glutamate receptors (CP-AMPARs.) We showed that a selective blocker of CP-AMPARs – the anandamide compound IEM-1460 – reduces synaptopathy caused by application of the glutamate agonist kainic acid to cochlear explants in vitro. We further showed that IEM-1460 inhibits KA-dependent Ca2+ entry into spiral ganglion neurons in vitro. Most significantly, we have used physiological measures – auditory brainstem response threshold and amplitude – and direct counting of synapses in confocal microscope images to demonstrate essentially complete prevention of synaptopathy and hearing impairment in noise-exposed mice without significant elevation of normal hearing threshold. This suggests that selective CP-AMPAR blockers such as IEM-1460 could be effective in protecting cochlear synapses from noise-induced synaptopathy and preventing the consequent hearing impairment.

15. SUBJECT TERMS

Calcium-Permeable AMPA Receptors, Cochlea, Excitotoxicity, Glutamate Receptor, Noise-Induced Hearing Loss, Organotypic Culture, Spiral Ganglion Neuron, Synapse, Synaptopathy

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INTRODUCTION

Moderate noise not loud enough to destroy auditory sensory cells (hair cells) and cause profound deafness still suffices to cause a significant hearing impairment by destroying synapses between hair cells and cochlear (spiral ganglion) neurons. This noise-induced "synaptopathy" can result in tinnitus and poor speech comprehension in a noisy background, common problem in military veterans and others exposed to noise. Synaptopathy is the result of excitotoxic trauma due to glutamate released from the hair cells. Excitotoxic trauma damages the postsynaptic cell by causing entry of Ca^{2+} ions. In the case of synaptopathy, our studies supported by this grant have identified the route of Ca^{2+} entry as via Ca^{2+}-permeable AMPA-type glutamate receptors (CP-AMPARs.) We accomplished this by showing that a selective blocker of CP-AMPARs – the anandamide compound IEM-1460 – reduces synaptopathy caused by application of the glutamate agonist kainic acid to cochlear explants in vitro. We further showed that IEM-1460 inhibits KA-dependent Ca^{2+} entry into spiral ganglion neurons in vitro. This suggests that selective CP-AMPAR blockers such as IEM-1460 could be effective in protecting cochlear synapses from noise-induced synaptopathy and preventing the consequent hearing impairment. The principal accomplishment of this past year has been to provide substantial evidence that this is the case. We have used physiological measures – auditory brainstem response threshold and amplitude – and direct counting of synapses in confocal microscope images to demonstrate essentially complete prevention of noise-induced synaptopathy and hearing impairment without significant elevation of normal hearing threshold. A second major accomplishment has been to refine our in vitro protocol to be a much more accurate model of in vivo noise damage to synapses.

KEYWORDS

Anandamide

Auditory Brainstem Response

Calcium Ion

Calcium-Permeable AMPA Receptors

Cochlea

Excitotoxicity

Glutamate Agonist

Glutamate Receptor

Hair Cell

Hearing Threshold

Noise-Induced Hearing Loss

Organotypic Culture

Spiral Ganglion Neuron

Synapse

Synaptopathy

MAJOR GOALS

1) **Assessment of the protective effect of IEM-1460 delivered by intracochlear perfusion.**
2) **Assessment of the ability of IEM-1460 to prevent excitotoxic damage to cochlear synapses in vitro.**
3) **Immunohistochemical determination of GluA2 location in control and kainate-exposed cochlear explants.**

ACCOMPLISHMENTS

1) Assessment of the protective effect of IEM-1460 delivered by intracochlear perfusion.

Specific objectives *(starting from month 3 and extending through the entire first year):*

a) *Refinement of the experimental protocol* to establish the appropropriate noise level for these experiments and achieve sufficient expertise in the surgery that at least half of the mice operated will not experience a significant elevation of hearing threshold that would render them unsuitable for tests of protection against noise damage. The reason for this concern is that if trauma from the surgery itself elevates hearing threshold – i.e., reduces hearing sensitivity – that in and of itself would reduce adverse effects of noise, but not in a way desirable for human therapy!

b) *Determine whether IEM-1460 affects hearing at normal sound levels*. IEM-1460 blocks glutamate receptors used for synaptic transmission at the hair cell to spiral ganglion neuron (SGN) synapse. It is therefore conceivable that IEM-1460 would affect normal hearing. Nevertheless, we predict that normal hearing would not be affected. GluA2 is present in SGNs and is present at synapses (see Goal 3, below.) Because the presence of a GluA2 subunit in a glutamate receptor renders it Ca^{2+}-impermeable and so not blocked by IEM-1460, we reason that these receptors can mediate synaptic transmission. The fraction of glutamate receptors that lack a GluA2 subunit are, presumably, those that are responsible for the Ca^{2+} influx and synaptopathy. However, there may not be enough of them to make a significant contribution to normal hearing at moderate sound levels.

c) *Determine definitively whether IEM-1460 prevents synaptopathy*. We consider assessment of the protective effect of IEM-1460 *in vivo* to be the most important goal of this project as this is most likely to lead directly to therapies usable in humans.

Methodology

Assessment of protection by IEM-1460 against noise damage to synapses in vivo:

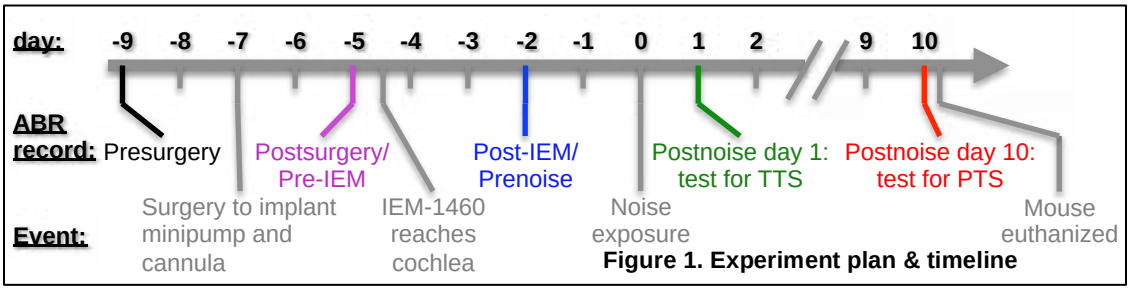

Figure 1. Experiment plan & timeline

The basic experimental plan is diagrammed above. The CBA/CaJ mice are surgically implanted with a minipump-cannula system providing IEM-1460 or control substances to the cochlea, as described below. After this, the mice are exposed to a moderate noise that destroys synapses on inner hair cells (IHCs) but spares the hair cells themselves. The consequences of the noise are assessed by ABR and immunohistochemistry. The purpose of the five separate auditory brainstem response (ABR) recordings at the indicated times is to ensure that the surgery itself does not impair hearing significantly (in which case the mouse has to be excluded from the study), that IEM-1460 does not significantly affect hearing, that the noise exposure is sufficient to cause a temporary threshold shift (TTS) in all mice, that there is no permanent threshold shift in any mice, and to test the ability of IEM-1460 to prevent a permanent decrease in ABR amplitude otherwise caused by the moderate noise exposure and to test the ability of IEM-1460 to prevent a loss of synapses otherwise caused by the moderate noise exposure.

The minipump we use is an Alzet model 2004 28-day pump with a nominal flow rate of 0.25 µl/hour. The exact flow rate for each individual pump is provided with the pump. The cannula (inner diameter 0.03 in) is filled with artificial perilymph (AP). The cannula length is calculated so that the minipump contents – either AP or 0.5 mM IEM-1460 in AP – reach the end of the cannula and begin to enter the cochlea 60 h after the surgical implantation (≈4.6 cm). The surgery to implant the minipump and cannula is done one week prior to noise exposure. Two days prior to surgery a **presurgery** ABR record is made to establish the baseline for each mouse. After allowing two days for recovery from the surgery, a **postsurgery** ABR is obtained to determine whether the surgery has seriously impaired hearing.

We allow an additional 60 h for the IEM-1460 to diffuse into the cochlea. At that time, a **post-IEM/prenoise** ABR is recorded to determine whether the IEM-1460 itself has affected hearing.

Noise exposure is 2 d after the prenoise ABR to allow time for recovery from the anesthesia. Temporary threshold shift (TTS) is determined by ABR on the day following noise exposure (**postnoise day 1**). We allow 10-14 days for recovery then test ABR: **postnoise day 10**. This allows us to determine whether there has been a permanent threshold shift (PTS.)

Drug administration: IEM-1460 in artificial perilymph (AP) is infused into the left cochlea of mice; the right ear is the unoperated control. The mini-osmotic pump (Model 2004, Alzet Osmotic Pumps) is connected to the cochlea by a series of three types of tubing of successively decreasing diameter. Most of the length is a polyethylene catheter (PE-60, I.D 0.03”, **Durect Corp.**); the length is calculated based on the pump flow rate to provide a 60 h delay before the minipump contents reach the cochlea. A polyimide tubing (Part number 95720-00, I.D 0.0049”, Cole-Parmer) is inserted into the round window (RW) and is connected to the main catheter by few mm length of polyurethane tubing (BB520-25, I.D 0.012”, Scientific Commodities, Inc.). A key control is to perform the surgery and subsequent procedures in exactly the way described here but to have *AP only* in the minipump with no IEM-1460. This controls for nonspecific effects of surgery and intracochlear perfusion.

Surgery to expose the round window (RW) of the mouse: The mouse is anesthetized and an incision made behind the left ear. The RW is exposed through a dorsal approach. After the bony wall is drilled off carefully with a micro-diamond burr, the RW can be directly accessed. The polyimide tubing is inserted 0.5-1 mm into the RW. As the tubing is inserted, the perfusion catheter is fixed to the bone with tissue glue, thereby sealing the fenestration. The mini-pump is put under the skin in the lower back. The incision is then sutured.

Noise exposure: We use a 2 hr noise exposure of 100 dB SPL, 8-16 kHz. Our protocol is as follows: Two mice are held awake and unrestrained in a small iron-wire cage (one mouse per cage), positioned head-to-head under the center axis of the speaker within a custom-made sound-proof chamber. Instruments for generating and controlling noise exposure include RZ6 multi I/O processor (Tucker-Davis Technologies, Inc.), a high frequency power amplifier (IPR-1600 DSP, Peavey Electronics Corporation), and a high frequency loudspeaker (Beyma driver CP21F, 1” HF slot tweeter, Carrer del Pont Sec.) The noise level was monitored with a ¼” condenser microphone (Model 7017, ACO Pacific, Inc.) placed at the center of the space between the two animals at the approximate level of the animals' ears. The variation of the noise level across the animals’ ears and across time is <1 dB.

ABR:

ABR recording: Under anesthesia with the mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg), alternative responses are recorded from 90 dB SPL to 10 dB below the threshold level in 10 dB descending steps. These are used to plot the wave I amplitude as a function of sound intensity for 8, 16, and 32 kHz. (Wave I is the ABR component corresponding to activity in the spiral ganglion neurons.) Near the threshold level, an additional descending and an ascending series of recordings are made in 5 dB steps to more accurately determine the threshold. The ABR threshold is defined as the lowest stimulus level that evoked a repeatable waveform based on an identifiable ABR wave I.

Instruments: RZ6 multi I/O processor, RA4PA 4 channel preamplifier, and MF1 speaker (Tucker-Davis Technologies, Inc.), a custom-made sound-proof chamber. *Operating software*: BioSigRZ (Version 5.6, Tucker-Davis Technologies, Inc.).

Acoustic stimuli: Tone-pips with duration of 5 ms and gated time of 0.5 ms, presented at rate of 21/s and at frequencies of 8, 16, 32 kHz, alternative polarities. Sound is delivered to the external auditory meatus of a mouse through a custom-made insertion tube which connected to the MF1 speaker earphone via a 10 cm polyethylene tube.

Recording electrode configuration: An active needle electrode is placed at the midline of the vertex of the skull, a reference electrode at the ipsilateral mastoid areas and a ground electrode at the low back area.

Recording parameters: The acquisition time is 12 ms, at sampling rate of 25,000/s. The high-pass filter is set at 3000 Hz, the low-pass filer at 100 Hz. The signals are averaged by 128-512 sweeps.

Histology and imaging:

Dissection: The mice are euthanized immediately after the final ABR (day 10-14). The mouse is anesthetized and decapitated. The initial dissection is done in 4°C PBS within 5 min for each ear. The bony shell of the cochlea is largely removed to expose the cochlear turns. The cochlea is then fixed in 4% PFA for 12 min and then transferred to 0.12 mM EDTA for decalcification at 4°C for 48 hours. After decalcification, further dissection is done to expose the basilar membrane. The cochlear tissue is permeabilized with 1% Triton in PBS for 1 h at room temperature, washed 3x with 0.1% Triton in PBS, then blocked in antibody blocking buffer 5% horse serum / 0.1% bovine serum albumin / 0.1% Triton / 0.02% NaN_3 for 60 min at room temperature.

Immunostaining: The hair cells are immunolabeled with combined anti-myosin VI and anti-myosin VIIA to verify that the noise exposure did not destroy hair cells. Postsynaptic densities (PSDs) and presynaptic ribbons are immunolabeled, respectively, with anti-PSD95 and anti-CtBP2.

Imaging: The organ of Corti is removed, typically in three pieces, and fixed on a cover slip. Imaging is currently with a confocal microscope. Low magnification images are obtained first using a 10x objective. These are used to align the pieces of organ of Corti to the mouse frequency place map in ImageJ. Higher magnification images are captured at the 8, 16, and 32 kHz locations. The image planes (z-slices) are captured at a spacing of 0.4 µm along the z-axis to construct the 3-dimensional image stacks.

Quantitative analysis: Gaps in the hair cell rows are counted to assess hair cell survival. *A synapse is defined as a co-localized PSD and ribbon*. Synapses are counted in the confocal image stacks with an optical disector technique. The total number of synapses on IHCs in each stack is counted and then divided by the number of IHCs to determine Synapses/IHC.

Significant Results

a) Refinement of the experimental protocol

For the purposes of this project, which is focused on noise-induced synaptopathy, the noise exposure must be sufficient to cause damage to synapses but not to the hair cells. As assessed by auditory brainstem response (ABR), the criteria are that the surgery should cause a threshold shift of <10 dB, there would have to be a significant temporary threshold shift (TTS) – empirically, on the order 35-40 dB – but no permanent threshold shift (PTS.) A smaller TTS would indicate that the noise exposure was inadequate to cause any significant trauma, presumably because of surgical trauma. A significant PTS would indicate hair cell damage and the mouse would have to be excluded from the study. (For the purposes of this report, only wave I of the ABR is considered; the later waves are outside of the cochlea and not directly relevant to this study.) For the surgery to be considered successful, the subsequent noise exposure should be able to cause a significant TTS but no permanent threshold shift PTS.

As shown in Fig. 2A, in the mice used in these experiments, the threshold shift due to surgery is <10 dB and typically ≈5 dB. This appears to be relatively small but its significance can be assessed by determining the effect on TTS. As shown in Fig. 2A, TTS in the left (operated) ear is comparable to TTS in the right (unoperated) ear and, in both cases is >35 dB. This indicates that the small post-surgery threshold shift does not affect sensitivity to noise. We could achieve this is about half of the mice used for these experiments, the remaining were excluded.

b) Does IEM-1460 affect hearing at normal sound levels?

Fig. 2A also shows that there is no further change in threshold from the post-surgery ABR measurement at -3 days to the pre-noise measurement at -1 days even though the IEM-1460 reaches the cochlea a day prior to the pre-noise measurement. This shows that IEM-1460 itself does not affect hearing threshold. Moreover, as shown in Figs. 3-5, there is no significant difference in ABR measures between ears receiving IEM-1460 and those receiving only artificial perilymph (AP), further indicating that IEM-1460 does not affect normal hearing.

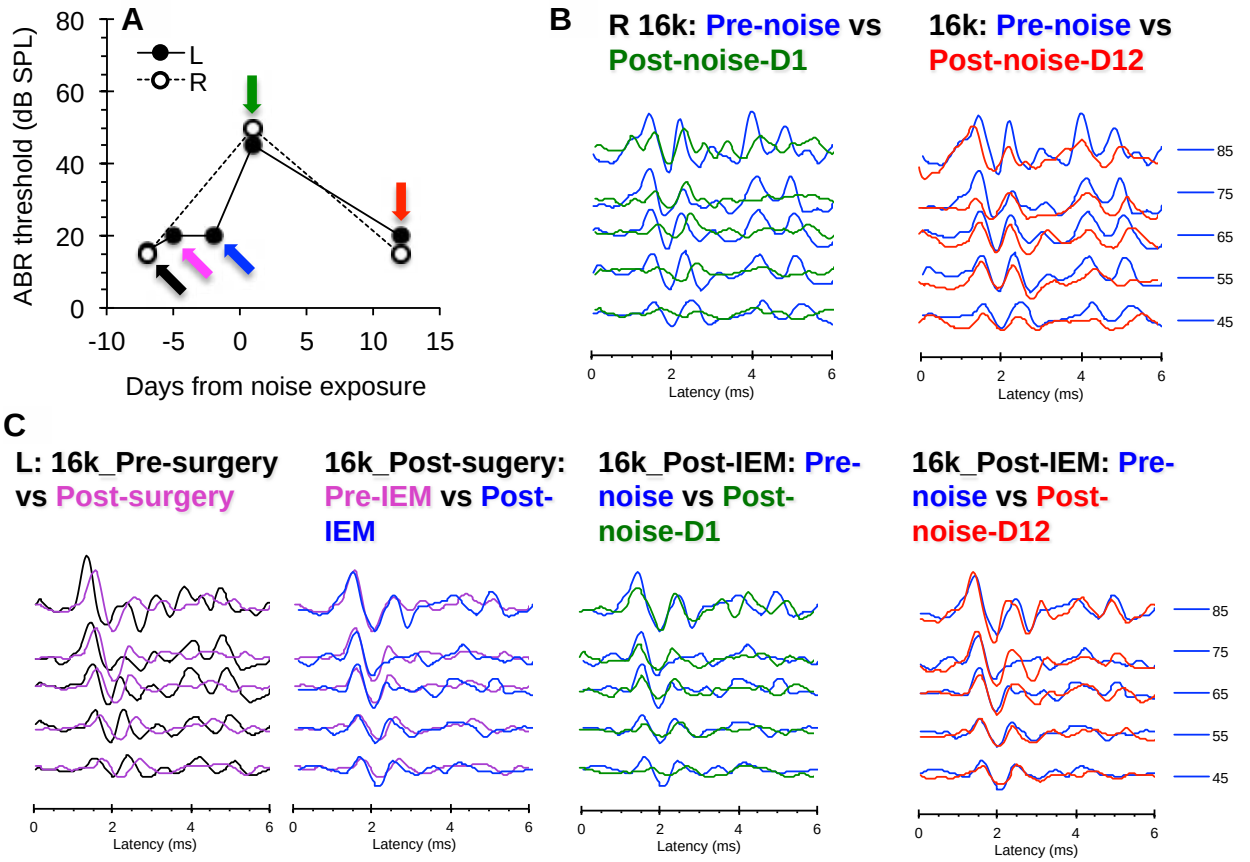

Figure 2. ABR Thresholds summary: ABR threshold measurements made at 16 kHz for each of the timepoints in Figure 1. All of the mice were exposed to identical noise on day 0. The left (**L**) ear was operated on to intracochlearly infuse IEM-1460; the right (**R**) ear was not operated but received the same noise exposure. **A**. **ABR thresholds for 16 kHz tone pips.** N = 11. These data are abstracted from more detailed threshold results shown in Fig. 3. Each ABR measurement is marked by an arrow color-coded to match the timeline in Figure 1, as follows: –9 days, presurgery control (normal baseline); –3 days, post-surgery control; –1 day, control for any possible effect of IEM-1460 on hearing; +1 day, test for temporary threshold shift; +10-14 days, test for permanent threshold shift. These measurements confirm that the surgery caused only a small, <10 dB, threshold increase ●. The IEM-1460 itself caused no change in hearing threshold ●. More significantly, response of the operated ear to noise was similar to that of the control unoperated ear: a TTS >35 dB ● and no PTS ●. Representative ABR waveforms from the right (unoperated) ear (**B**) and the left (operated) ear (**C**) are shown, also color-coded to indicate when the measurement was made. D1: 1 day post-noise; D12: 12 days post-noise

c) Determine definitively whether IEM-1460 prevents synaptopathy.

Having established a suitable surgical approach and shown that IEM-1460 does not significantly affect normal hearing, we ask whether IEM-1460 is protective against noise-induced synaptopathy, this being the most essential goal of the project. The data we have obtained in this reporting period (Oct 2014 - Sep 2015) strongly support this being the case. We have shown this by physiological measures – i.e., ABR – and by direct counting of synapses. We have acquired all of the data for analysis of ABR and and synapse numbers at three different cochlear locations, 8, 16, and 32 kHz, are quantitatively analyzing them, and are starting to write the manuscript. The manuscript will also include the data obtained from studies of excitoxicity in cochlear explant cultures, summarized here starting on page 12, as well as data showing blockade of kainic acid-induced Ca^{2+} entry into SGNs by IEM-1460. The latter data was largely obtained prior to this award and was described in the original grant application so is not discussed in this report. Although we have not yet completed all of the quantitative analysis for ABR amplitudes and synapse numbers at the 8 kHz and 32 kHz locations, we have done so for the 16 kHz location. We expedited the counts for the 16 kHz location because, as the upper bound of the frequency range used

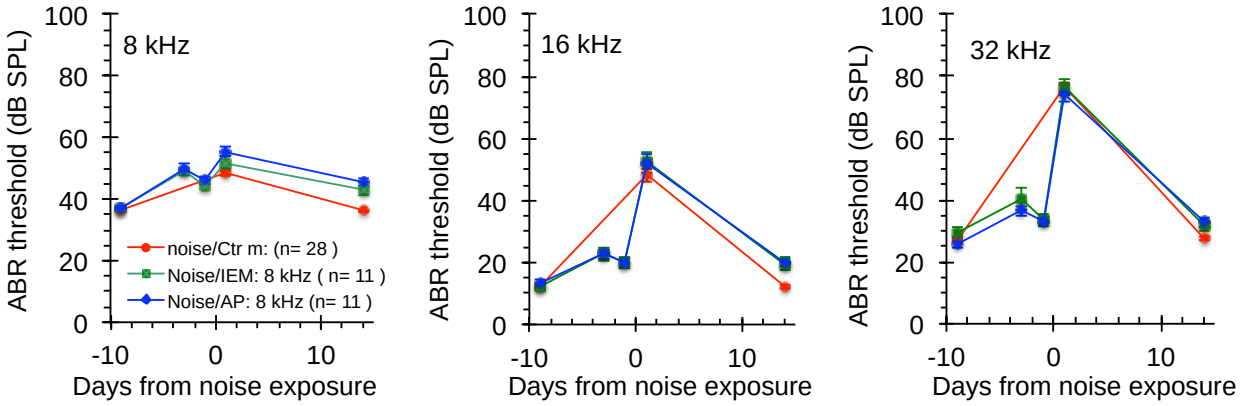

Figure 3. ABR threshold measurements (mean ± SEM) at 8, 16, and 32 kHz for each of the timepoints in Fig. 1 (and accordingly color-coded: –9 days, presurgery control (normal baseline); –3 days, post-surgery control; –1 day, control for any possible effect of IEM-1460 on hearing; +1 day, test for temporary threshold shift; +10-14 days, test for permanent threshold shift. All of the mice were exposed to identical noise on day 0. In one ear of each mouse, the cochlea was accessed surgically prior to the noise exposure to infuse either artificial perilymph (Noise/AP) or artificial perilymph with 0.5 mM IEM-1460 (Noise/IEM). The contralateral ear was control untreated (noise/Ctr.)

for the noise exposure – 8-16 kHz octave band – it is the region of the cochlea at which synaptopathy is expected to be most pronounced. These data are presented here.

The characteristics of synaptopathy are *(1) no PTS but a permanent reduction in ABR wave I amplitude* and *(2) a reduced number of afferent synapses on inner hair cells*. ABR thresholds are shown in Fig. 3 at 8, 16, and 32 kHz locations (these threshold data are complete) for noise-exposed ears intracochlearly perfused with IEM-1460 in AP, with AP only, or unoperated. These data establish that, in all ears exposed for two hours to 100 dB SPL 8-16 kHz octave band noise, there is a TTS but no significant PTS. The data further show that the degree of TTS is not significantly different among the three different conditions indicating that neither the surgery nor the IEM-1460 affects sensitivity of the cochlea to noise.

ABR amplitude: Moderate noise that causes TTS but not PTS can result in a permanent reduction in ABR wave I amplitude. As shown in Figs. 4 and 5, this is the case for the noise exposure used here, validating this aspect of the experimental protocol. *The data further show that blockade of Ca^{2+}-permeable AMPA receptors by intracochlear perfusion of IEM-1460 prevents the reduction in wave I amplitude.* Comparison of the prenoise (blue) to the 14 d postnoise (red) growth curves for the Noise/IEM

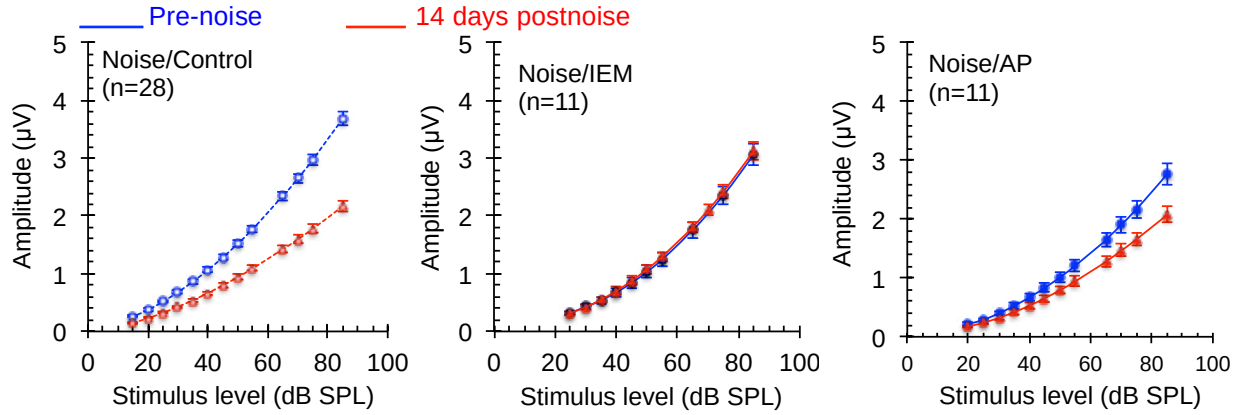

Figure 4. ABR wave I amplitude measurements for 16 kHz tone pips at indicated sound levels. Shown are means ± SD. The curves were constructed by fitting the data (by least squares) to a second-order polynomial. These were then compared pairwise using extra sum-of-squares F-tests to determine whether differences among the curves are significant. **Noise/Control:** Wave I amplitude measurements from control noise-exposed unoperated ears, pre- and postnoise are significantly different, $p<0.01$ for F-test comparison of data fitted to a first-order polynomial. **Noise/IEM:** Wave I amplitude measurements from noise-exposed ears perfused intracochlearly with 0.05 mM IEM-1460 in AP, pre- and postnoise are not significantly different. **Noise/vehicle only:** Wave I amplitude measurements from noise-exposed ears perfused intracochlearly with AP, pre- and postnoise are are significantly different, $p<0.01$. The postnoise values from the **Noise/vehicle only** ears are *not* significantly different from from postnoise values from the **Noise/Control** ears.

ears demonstrates the complete recovery of ABR wave I amplitude, in contrast to the decreased amplitude of the 14 d postnoise growth curve for the Noise/Control ears. However, the Noise/AP ears showed no prevention of the noise-induced amplitude reduction. The results from these ears were similar to those of the Noise/Control ears and significantly different ($p<0.01$) from the results of the Noise/IEM ears. These results confirm that *the protective effect of intracochlear perfusion of IEM-1460 is due to the IEM-1460.* This key result is also shown in Fig. 5, a completed statistical analysis of the decline in ABR wave I amplitude for all data obtained for 16 kHz stimuli.

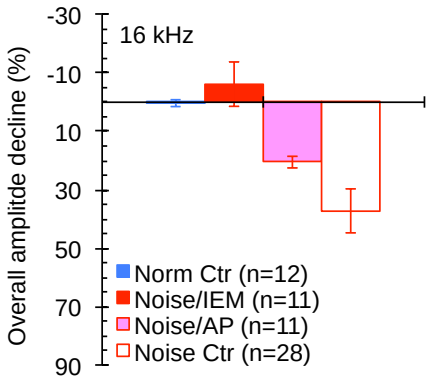

Figure 5. Decline in ABR wave I amplitude after a 2 hr exposure to 100 dB SPL 8-16 kHz octave band noise. Shown are means ±SD. Differences are highly significant:
One-way ANOVA, $F(3, 58) = 40.45$, $p < 0.0001$.
Tukey's multiple comparison test:
Norm Ctr vs. IEM: P>0.05
Norm Ctr vs. AP: P<0.001
Norm Ctr vs. Noise ctr: P<0.0001
IEM vs. AP: P<0.0001
IEM vs. Noise ctr: P<0.0001
AP vs. Noise ctr: P<0.001

Synapse number: Representative images of synapses are shown in Figs. 6 and 7. The results shown in Figs. 4 and 5 imply that cochleae receiving IEM-1460 would not exhibit noise-induced synapse loss but that control unoperated cochleae and cochleae receiving AP only would show comparable synapse loss. Preliminary data from synapse counts (described above in Methodology) indicate that this is indeed the case (Fig. 8.) Completed data from the 16 kHz location (Fig. 8B) confirm that IEM-1460 treatment prevents synaptopathy: in noise-exposed cochleae perfused with IEM-1460, synapse number is the same as in ears not exposed to noise. In contrast, in noise-exposed cochleae perfused with AP only, synapse number is the same as in unoperated noise-exposed cochleae.ears not exposed to noise. *These data provide support for the conclusion that IEM-1460 effectively protects against noise-induced synaptopathy*.

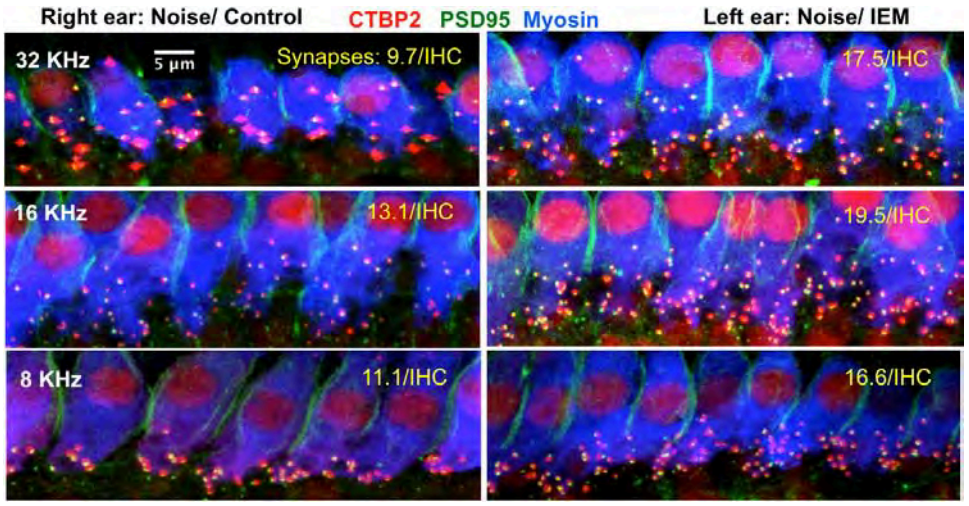

Figure 6. Representative examples of three different cochlear locations – 8, 16, and 32 kHz – from noise-exposed unoperated control (left panels) or IEM-1460-treated (right panels) ears. These are projections onto a plane ("z-projections") of three dimensional confocal image stacks. Labeling is as described in Methodology: CTBP2 (red) for ribbons (and hair cell nuclei), PSD95 (green) for postsynaptic densities, and myosin 7a (blue) for hair cells. Synapse counts – i.e., number of synapses/inner hair cell (IHC) – are shown in yellow for each image. Scale bar (white, upper left panel) is 5 µm.

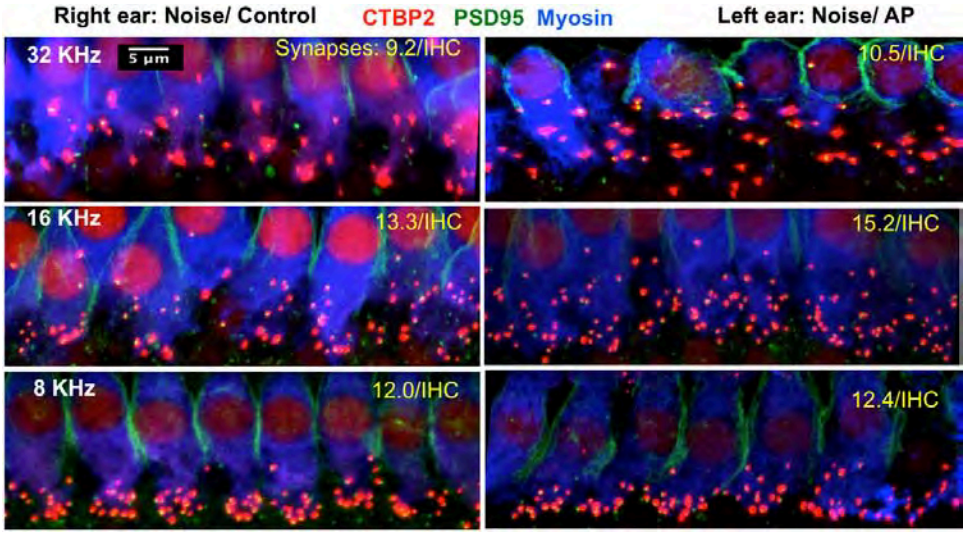

Figure 7. Representative examples of three different cochlear locations – 8, 16, and 32 kHz – from noise-exposed unoperated control (left panels) or ears operated but perfused just with AP (right panels.) Images were prepared as for Figure 6.

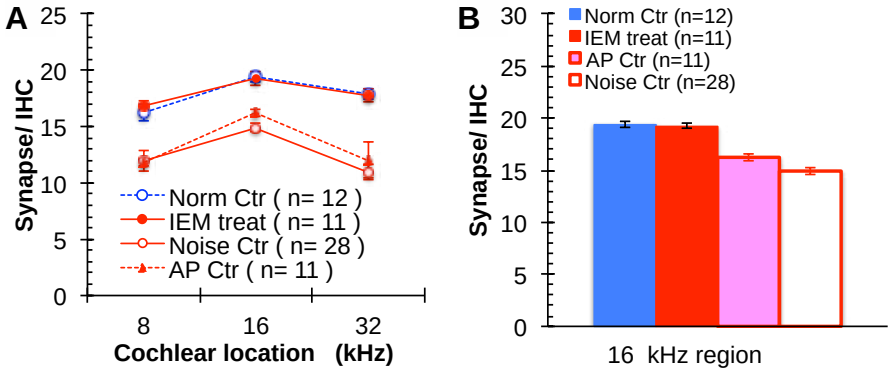

Figure 8. A. Number of synapses (means ± SD) surviving after noise exposure in vivo for the indicated number of cochleae at three cochlear locations, 8, 16, and 32 kHz. Cochleae are either normal **Control** not exposed to noise, noise-exposed perfused with IEM-1460 (**IEM treat**), noise-exposed unoperated (**Noise Ctr**), or noise-exposed perfused with artificial perilymph (**AP Ctr**) There is no significant difference between **AP Ctr** and **Noise Ctr** at all three locations nor is there a significant difference between **Norm Ctr** and **IEM treat** at all three locations. However, both **AP Ctr** and **Noise Ctr** are significantly different ($p<0.0001$) from both **Norm Ctr** and **IEM treat** at all three locations. Statistics: ANOVA, Holm-Sidak correction for multiple comparisons. **B.** Number of synapses (means ± SD) surviving after noise exposure in vivo for the indicated number of cochleae at the 16 kHz location. Differences are highly significant:
One-way ANOVA: $F(3, 58) = 44.75$, $p < 0.0001$
Turkey's multiple comparison test:

- Norm Ctr vs. IEM: $P>0.05$
- Norm Ctr vs. AP: $P<0.0001$
- Norm Ctr vs. Noise ctr: $P<0.0001$
- IEM vs. AP: $P<0.0001$
- IEM vs. Noise ctr: $P<0.0001$
- AP vs. Noise ctr: $P<0.05$

2) Assessment of the ability of IEM-1460 to prevent excitotoxic damage to cochlear synapses in vitro.

In preliminary experiments performed prior to the beginning of this reporting period it was noted that IEM-1460 did not full prevent excitotoxic damage to synapses in an in vitro model of synaptopathy: cochlear explant cultures exposed to the glutamate agonist kainic acid (KA.) This could be due to the possibility that excitotoxic damage is caused only partly by Ca^{2+} entry and partly by a different cause. Here we test the possibility that osmotic stress is responsible in part for excitotoxic damage to synapses. Alternatively, it may be that the in vitro excitotoxic stress is exceptionally strong and is not an accurate model of noise exposure in vivo.

Methodology

Using neonatal (postnatal day 5, P5) rat cochleae, a portion of the organ of Corti and corresponding part of the spiral ganglion – these experiments use the middle of the cochlea – is transferred to a culture dish where it can be maintained for days. The organotypic explant culture maintains organ of Corti and associated spiral ganglion with cell-cell and synaptic contacts intact, qualitatively and quantitatively resembling the *in vivo* peripheral auditory system.

For excitotoxic trauma, the explants are exposed to KA at the indicated concentration for 2 hr (same time duration as the noise exposure *in vivo*.) Any protective agent(s), IEM-1460 and/or mannitol, are present 30 min in advance and throughout the KA exposure. The explants are fixed 8 h after KA exposure, labeled with antibodies to detect hair cells, presynaptic ribbons, and PSDs. The same antibodies are used as for the *in vivo* experiments: for hair cells, anti-myosin 6 and/or 7A; for SGNs anti-high-molecular neurofilament (NF200) and/or NF150 with β-III tubulin; for ribbons, anti-CtBP2 (which conveniently also labels hair cell nuclei) and for PSDs, anti-PSD95. The explants are imaged by confocal microscopy. Synapses, defined as a co-localized PSD and ribbon, are counted by the same procedure as for cochlear wholemounts from the in vivo experiments. We count PSDs in 2-3 segments/cochlea, each containing 8-9 IHCs, all from the middle of the cochlea to reduce variability due to physiological differences between apical and basal synapses. From these data, we calculate synapses/IHC.

Specific objectives *(first year):*

a) *Dose response to KA and IEM-1460 in vitro.* This tests the hypothesis that the in vitro excitotoxic stress we have used – 0.5 mM KA for two hours – does not accurately noise exposure in vivo and a lower concentration of KA might be more appropriate. This hypothesis is based on two observations made during this past year. First, the 0.5 mM KA exposure experiments typically resulted in a loss of >90% of the synapses while noise exposure causes a loss of about 25-30% of the synapses. Second, 0.5 mM IEM-1460 was able to rescue only a fraction of the synapses lost during a 2 hr exposure to 0.5 mM KA in vitro but appears able to entirely rescue all synapses lost during a 2 hr noise exposure in vivo. Thus, our objective here is to determine whether a lower concentration of KA might allow a better in vitro model for noise-induced synaptopathy and, in particular, whether IEM-1460 might be completely protective with a concentration of KA that mimics the extent of synapse loss in vivo in noise-exposed mice.

b) *Assess the ability of IEM-1460 and mannitol to improve synapse regeneration in vitro.* This tests the hypothesis that excitotoxic damage to synapses is due in part to osmotic stress and can be prevented with an osmoprotectant such as mannitol. If this is the case, then combined treatment with IEM-1460 and mannitol might allow better rescue of synapses than either alone.

Significant Results

a) Dose response to KA and IEM-1460 in vitro.

We have found that intracochlear perfusion of 0.5 mM IEM-1460 is completely protective against noise-induced synaptopathy. While obviously a very welcome result, it means that our earlier in vitro studies appeared to underestimate the efficacy of IEM-1460 in that 0.5 mM IEM-1460 did not completely prevent synapse loss caused by 0.5 mM KA. A potentially crucial difference between the in vivo and in vitro models is that in the former, not all synapses are lost, only about 20%-30%. In our earlier in vitro experiments, in which excitotoxic trauma was accomplished using 0.5 mM kainate, >90% of the synapses were lost. We reasoned that this was because the high concentration of KA causes greater synapse loss

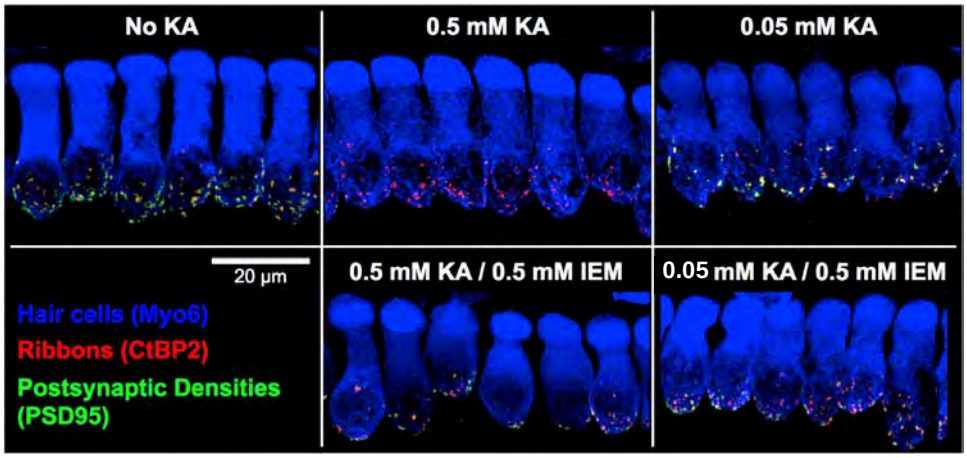

Figure 9. Representative examples of organotypic cochlear explant cultures exposed to either 0.05 mM or 0.5 mM KA in the presence or absence of 0.5 mM IEM-1460, as well as a control culture not exposed to KA. These are projections onto a plane ("z-projections") of three dimensional confocal image stacks. Labeling is as described in Methodology: CTBP2 (red) for ribbons (and hair cell nuclei), PSD95 (green) for postsynaptic densities, and myosin 7a (blue) for hair cells. Synapse counts – i.e., number of synapses/inner hair cell (IHC) – are shown in yellow for each image. Scale bar = 20 µm.

than does noise in vivo. That is, the in vitro trauma is unrealistically severe and underestimates the ability of IEM-1460 to protect synapses.

If the above hypothesis is correct then IEM-1460 should be completely protective against excitotoxic damage to synapses in vitro under conditions in which the damage to synapses is comparable to that caused by noise exposure in vivo. We titered the KA to identify a concentration that causes synapse loss comparable to that caused by moderate noise in vivo, 20%-30% of synapses. Representative images are shown in Fig. 9 and demonstrate that reducing the KA concentration from 0.5 mM to 0.05 mM decreases the number of PSDs lost and 0.5 mM IEM-1460 appears to be more effective as a protective agent.

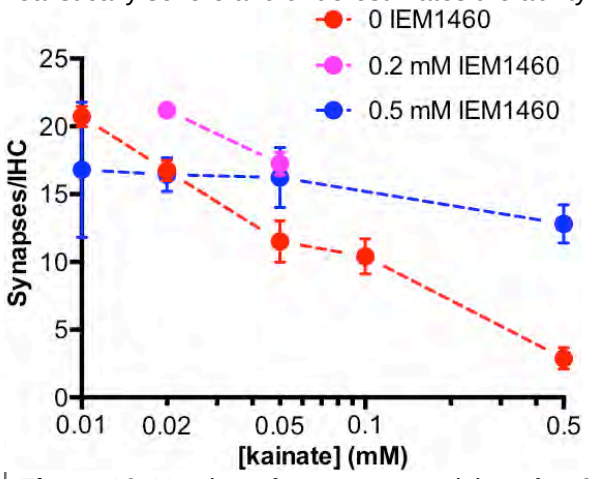

Figure 10. Number of synapses surviving after 2 hr exposure to the indicated concentrations of kainate and the indicated concentrations of IEM-1460. Shown are means ± SEM for indicated number (n) of cochleae.

Counts of synapses were made near the middle of the cochlea, approximately corresponding to the 16 kHz location in the mouse. The data, shown in Fig. 10, indicate that 0.02-0.05 mM KA is an appropriate condition for this experiment. That is, exposure to these KA concentrations causes a synapse loss compartable to that caused by noise in vivo. With these KA concentrations, but not with 0.5 mM KA, we observe a *complete or near-complete rescue of synapses by IEM-1460*, comparable to what we have observed in vivo. These data have allowed us to create a more accurate in vitro model of excitotoxicity that can be used for future studies.

The data shown in Fig. 10 indicate a possibility that 0.2 mM IEM-1460 is better at protecting synapses than is 0.5 mM IEM-1460 and that 0.5 mM IEM-1460 may even have a small adverse effect on synapse number. Such an adverse effect, which is not statistically significant, has not observed in vivo. Possibly, the IEM-1460 is diluted when entering the cochlea and is actually at a lower concentration than in the cannula. Certainly, these preliminary results from the in vitro model will be followed up in the next year.

b) Assess the ability of IEM-1460 and mannitol to improve synapse regeneration in vitro.

The original rationale for assessing osmoprotectants such as mannitol in combination with IEM-1460 to prevent synaptopathy was that it appeared that IEM-1460 alone was inadequate to completely prevent

synaptopathy. As the project has developed over the past year, it appears that IEM-1460 is able to completely prevent noise-induced synaptopathy in vivo and also to prevent excitoxic damage to synapses in vitro provided that the excitotoxic trauma is titered to mimic the level of damage caused by noise in vivo. Thus, it might be the case that addition of mannitol will be unable to provide any additional protection and effort expended on investigating osmoprotection might be better spent on investigating synapse regeneration (i.e., acceleration and expansion of Task 5.) In this reporting period we have begun testing that possibility. Preliminary results are shown in Figure 11. These data indicate that, while mannitol may have a small protective effect on its own (not significant in this study), co-treatment with IEM-1460 and mannitol does not improve on the protective effect of IEM-1460 alone. We must consider the possibility that our efforts and resources would best be directed toward the regeneration goals.

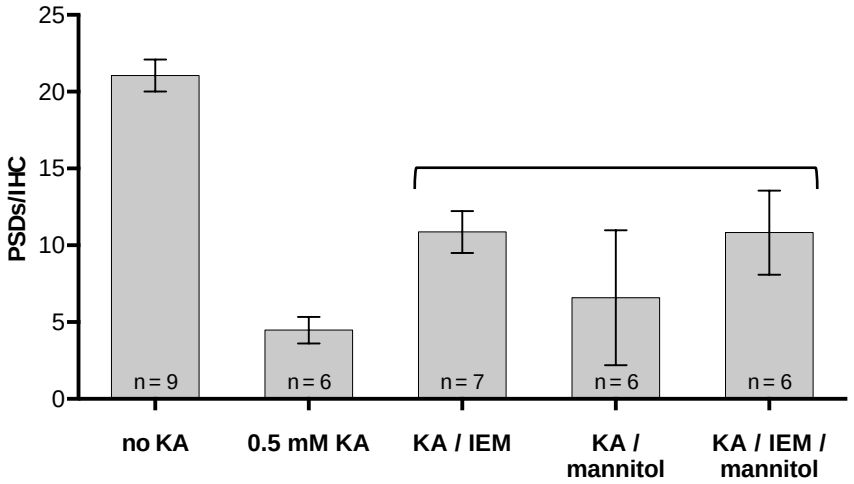

Figure 11. Synapse number (mean ± SEM) in the indicated number (n) of P5 rat cochlear explant cultures exposed to experimental conditions. In these combinations, [KA] = 0.5 mM, [IEM-1460] = 0.5 mM, and [mannitol] = 100 mM. Columns under the horizontal bar are significantly different from 0.5 mM KA and the no KA control but are not different from each other (ANOVA with Tukey's multiple comparisons test.)

3) Immunohistochemical determination of GluA2 location in control and KA-exposed cochlear explants.

Our observation that a blocker of Ca^{2+}-permeable AMPA receptors (CP-AMPARs) is protective against excitotoxicity seems paradoxical. AMPA-type glutamate receptors are tetramers that can contain any of the four GluA subunits (GluA1-4) expressed in a particular neuron. The presence of even a single GluA2 subunit in the tetramer renders that receptor impermeable to Ca^{2+}. SGNs express primarily GluA3, GluA4, and a small level of GluA2. GluA2 is definitely present at afferent synapses on IHCs leading to the question of how can there be CP-AMPARs if GluA2 is present. We considered three hypotheses: *(1)* not every synapse has AMPA receptors with GluA2 subunits; (2) all synapses have glutamate receptors with GluA2 subunits but the GluA2 is rapidly internalized during excitotoxic trauma leaving primarily CP-AMPARs on the surface; (3) all synapses have glutamate receptors with GluA2 subunits but many or most of the receptors lack a GluA2 subunit and are Ca^{2+}-permable. This latter hypothesis seems quite possible given that GluA2 is present at lower levels than GluA3 and GluA4 so the stoichiometry would predict that, at each synapse, there will be glutamate receptors that lack GluA2 and are Ca^{2+}-permeable among receptors that include a GluA2 subunit.

Specific objectives *(first year):*

To distinguish among these hypotheses we have initiated studies using immunofluorescent dual labeling of GluA2 and of PSD95, the latter being a marker of postsynaptic sites. The questions asked are whether GluA2 and PSD95 are colocalized, i.e., does every postsynaptic site contain GluA2, and whether this colocalization is maintained during exposure to KA in vitro (mimicking noise in vivo.)

Methodology

Cultures were prepared and exposed to 0.05 mM or 0.5 mM KA as summarized above in Goal 2. The cultures were fixed after brief exposures, 5 or 30 min in KA. Immunolabeling was also as above except that an anti-GluA2 antibody was included.

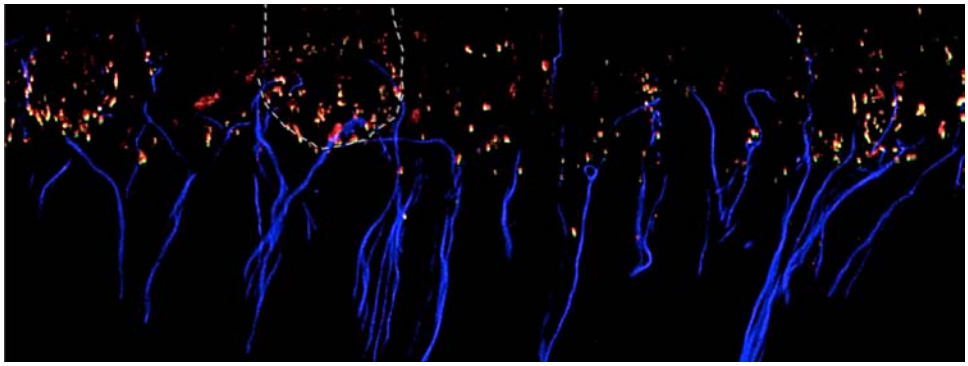

Figure 12. Representative example of an organotypic cochlear explant culture. This is a projection onto a plane ("z-projections") of a three dimensional confocal image stacks. Labeling: anti-neurofilament to detect SGN axons (blue), with anti-PSD95 to detect postsynaptic densities (green), and with anti-GluA2 (red). Colocalized PSD95 and GluA2 appears yellow. The hair cells were not immunolabeled – it was not necessary for this experiment – but were viewed in brightfield and the outline of a hair cell is indicated with a dashed line.

Significant Results

Our preliminary data do not support either of the first two hypotheses. Fig. 12 shows an example of an organotypic cochlear explant immunolabeled to detect colocalization of GluA2 and postsynaptic densities (PSD95 labeling.) In these preliminary experiments we have found 98% colocalization of GluA2 with PSD95, which argues against our first hypothesis. We also labeled GluA2 and PSD95 in explants exposed to different concentrations of KA for different time durations (Fig. 13.) While the number of synapses decreases with time, the degree of colocalization does not appear to vary significantly. This argues against the second hypothesis. These experiments need to be replicated and extended to additional timepoints to be certain but it does not seem likely that either of our first two hypotheses are likely. The third hypothesis will be tested by dual labeling of GluA2 with GluA3 or GluA4 antibodies to determine whether or not GluA2 always colocalizes with these other subunits within a synapse.

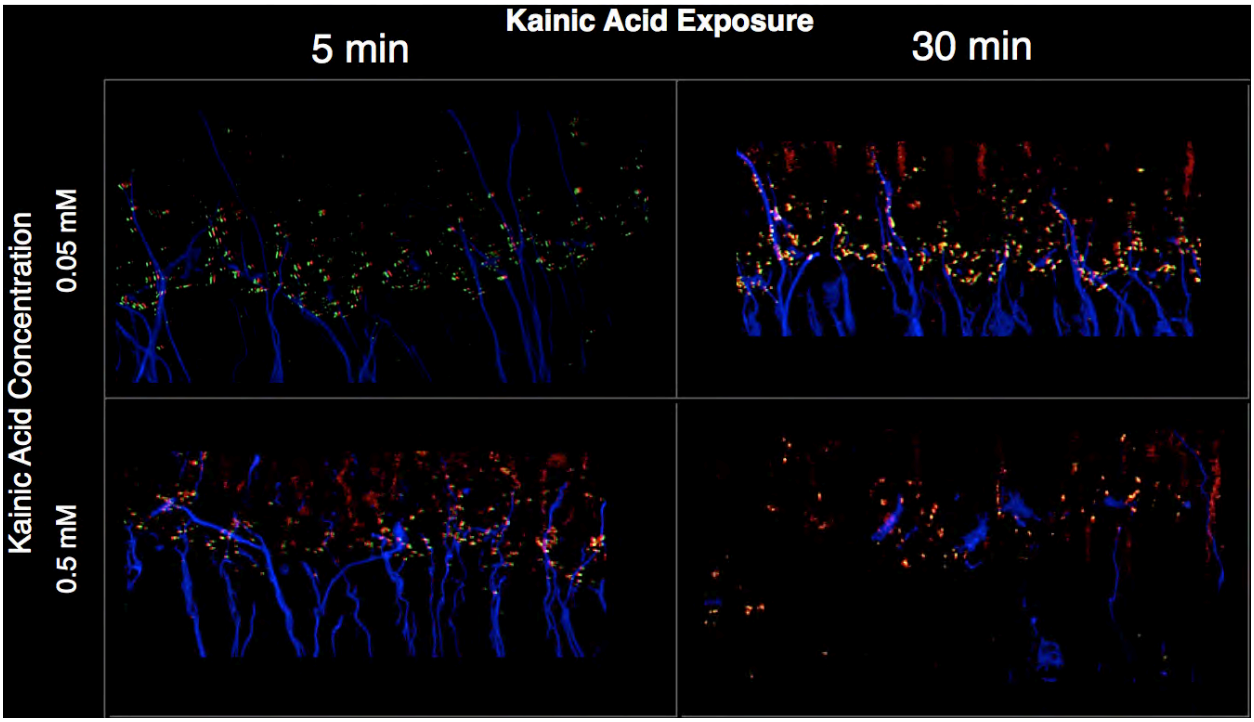

Figure 13. Representative examples of organotypic cochlear explant cultures exposed to either 0.05 mM or 0.5 mM KA for 5 min or 30 min. These are projections onto a plane ("z-projections") of three dimensional confocal image stacks. Labeling is as in Figure 12.

Training: A new graduate student Sriram Hemachandran has been trained in neural tissue culture techniques, microscopy, computer analysis of digital images.

Dissemination of Results: These results have been reported at the Association for Research in Otolaryngology 38th Annual MidWinter Meeting, Baltimore, MD, Feb 2015.

Future Plans. In the next reporting period, we plan to focus on the following main objectives:

- We will complete our quantitative analysis of the in vivo and in vitro data from the results reported here and complete and submit a manuscript on these results. This may involve repeating some in vitro experiments to increase the number of replicates to establish statistical certainty. We also plan to submit a patent application.
- Time permitting we will initiate studies of the effects on synapse regeneration in vitro of combining IEM-1460 protection and subsequent neurotrophic factor therapy.
- For the in vivo experiments, we have essentially completed the experiments necessary for a manuscript. In the next reporting period, we will initiate studies of the dose response to IEM-1460: determining the effectiveness of lower concentrations. Time permitting, we will initiate studies of alternative means to deliver IEM-1460 to the cochlea that are less invasive than a cannula. Specifically, we will apply gels, e.g., fibrin gels or hydrogels, to the round window that could allow slow release of the IEM-1460 over time.
- We will continue our investigation of the localization of GluA2 at cochlear synapses to understand how it is possible that synaptopathy appears to be largely or solely due to Ca^{2+}-permeable AMPA receptors. We will continue to use our in vitro model for these studies because of the speed by which multiple trials can be done but, time permitting, we will also investigate this question in vivo.

Impact:

Principal Discipline: The findings made in this reporting period have provided a new insight into the causes of noise damage to hearing. While avoiding noise is optimal, the findings being developed in this project may provide a means to prevent one of the most common types of noise-induced hearing impairment.

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Other Disciplines: Software we developed for quantitation of colocalized structures in digital images has been used by us to count synapses in microscope images but can be used for diverse purposes in analysis of digital images.

Technology Transfer: Nothing to report.

Society beyond science and technology: Nothing to report.

Changes:

We have not yet made any significant changes from the original proposal. However, given the apparent inability of the osmoprotectant mannitol to provide any significant additional protection beyond that afforded by IEM-1460 and given that IEM-1460 appears to be capable on its own of completely protecting synapses from noise exposure in vivo, we propose that the future goals of this project be changed to reduce further studies of mannitol and expand studies of the use of neurotrophic factors to induce synapse regeneration as a strategy for post-noise treatment.

Problems: In the July quarterly report we reported that our mouse colony had become infested with fur mites and would be treated with an avermectin compound, salemectin, for up to eight weeks. Because there are no studies on whether avermectin compounds affect noise damage, we could not use the mice for noise exposure experiments with IEM-1460 during the treatment period. This was an inconvenience but we were able to take some advantage of the situation. Avermectin compounds are widely used for

control of parasites in livestock as well as research animal housing. We felt it would be helpful to the auditory research community to know whether such compounds do affect hearing. Because we had control mice tested by ABR immediately prior to the beginning of the treatment and mice treated with salemectin, we compared them to determine whether the treatment made a difference. In fact, as shown in the figure below, salemectin did adversely affect hearing. Although there was no effect on normal thresholds, sensitivity to noise was increased. A 2 hr exposure to 100 dB SPL 8-16 kHz octave band noise does not typically cause a permanent threshold increase. However, in salemectin-treated mice a clear PTS is evident at 8, 16, and 32 kHz, particularly pronounced at 16 kHz. We are currently writing a manuscript that may be of use to the auditory research community as fur mite infestations followed by avermectin treatment are not uncommon.

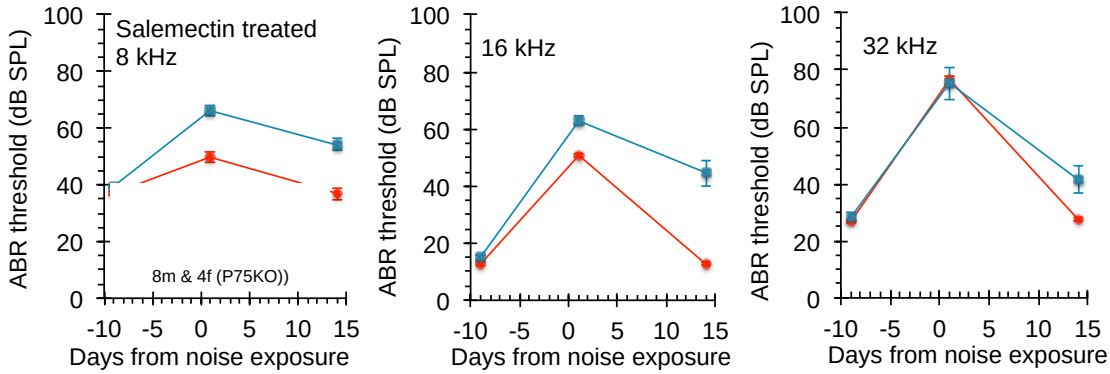


Figure 14. ABR wave I thresholds at 8, 16, and 32 kHz, prior to noise exposure, one day after noise exposure (to detect TTS) and 14 days after noise exposure (to detect PTS.) Red symbols are control mice (n=49, 36 male, 13 female) and blue symbols are salemectin-treated mice (n=12, 8 male, 4 female)

There are no other significant changes to report.

Products: Nothing to report.

Individuals who have worked on the project:

Name:	Steven Green
Project Role:	Principal Investigator
Nearest person month worked:	8
Contribution to Project:	Planning experiments; data analysis; software development
Other support	NIH, University of Iowa

Name:	Ning Hu
Project Role:	Research Scientist
Nearest person month worked:	12
Contribution to Project:	Planning experiments; mouse surgery; ABR measurement; data analysis; microscope imaging and analysis of digital images

Name:	Catherine Kane
Project Role:	Research Assistant
Nearest person month worked:	4
Contribution to Project:	Maintain animals; prepare organotypic cochlear explant cultures; microscope imaging; training students
Other support	NIH

Name:	Sriram Hemachandran
Project Role:	Graduate Student
Nearest person month worked:	8

Contribution to Project:	Microscope imaging and analysis of digital images; culture of organotypic cochlear explants and spiral ganglion neurons

Name:	Sepand Bafti
Project Role:	Undergraduate Student
Nearest person month worked:	4
Contribution to Project:	Software development; data analysis; in training for culture of organotypic cochlear explants

Changes in active other support: Nothing to report.

Special Reporting Requirements: The quad chart is appended.

There is no appendix.

Prevention of Noise Damage to Cochlear Synapses

W81XWH-14-1-0494

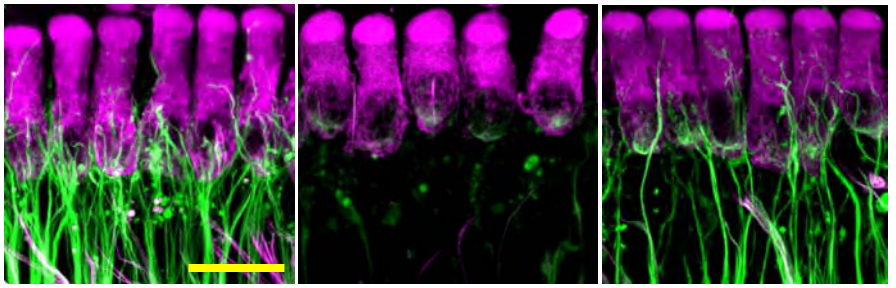
PI: Steven Green **Org:** University of Iowa **Award Amount:** $1,484,000

Study Aims

- Does blockade of CP-AMPARs in vivo prevent or reduce noise-induced synaptopathy
- Is there a cause of synaptopathy other than Ca^{2+} entry?

Approach

Two means for protection against damage to cochlear synapses will be assessed: (1) IEM1460, a selective blocker of Ca^{2+}-permeable AMPA-type glutamate receptors and (2) mannitol, an osmoprotectant. These agents will be initially assessed alone and in combination in an in vitro model that uses kainate to damage synapses and subsequently will be assessed in vivo in mice exposed to noise sufficient to damage cochlear synapses.



Control – no KA **0.5 mM KA x 2 hr** **KA & 0.2 mM IEM**

Images of cochlear explant cultures: inner hair cells (IHCs) labeled with anti-myosin 6 (magenta), spiral ganglion neuron (SGN) axons labeled with neurofilament-200 (green). Exposure to 0.5 mM kainic acid (KA) for 2 hr destroys IHC-SGN synapses and the distal ends of the axons. Inclusion of 0.2 mM IEM-1460 with the KA significantly reduces excitotoxic trauma to synapses preserving >60% of IHC-SGN contacts. Scale bar (yellow) = 20 µm.

Timeline and Cost

Activities / CY	14	15	16
In vitro quantitation of neuroprotection by IEM1460, mannitol vs. kainate			
In vitro quantitation of neuroprotection by IEM1460 & mannitol in combination			
Quantitation of protection of synapses vs. noise by IEM1460			
Quantitation of protection of synapses vs. noise by mannitol			
Quantitation of protection of synapses vs. noise by combined treatments			
Estimated Budget ($K)	**$600**	**$436**	**$448**

Goals/Milestones

CY14 Goals – In vitro studies of neuroprotective agents alone and in combination: protection vs. excitotoxic trauma

☐Quantify protection of synapses by IEM1460 and by mannitol

☐Quantify protection of synapses by drug combinations

CY15 Goal – In vivo studies of neuroprotective agents vs. noise

☐ Auditory brainstem response in mice exposed to noise: control, IEM-1460 treated, mannitol-treated; dose-responses

☐ Quantify protection of synapses by histological measures in noise-exposed mice treated with IEM 1460 or mannitol; dose-responses

CY16 Goal – In vivo studies of neuroprotective agents vs. noise

☐ Auditory brainstem response in mice exposed to noise with IEM 1460 or mannitol, continue dose-response, drug combinations

☐ Quantify protection of synapses by histological measures in noise-exposed mice treated with IEM 1460 or mannitol: continue dose-response, drug combinations