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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 Ca^{2+} ions. We have identified the route of Ca^{2+} entry as via Ca^{2+} -permeable AMPA-type glutamate receptors (CP-AMPA). These are a subset of glutamate receptors that lack the GluA2 subunit. We showed that a selective blocker of CP-AMPA – the anandamide compound IEM-1460 – is protective against excitotoxicity and noise-induced synaptopathy. For the latter result we perfused IEM-1460 directly into the cochlea. In this research period we have made three significant advances in understanding synaptopathy and protection from it. First, we have shown that female mice are significantly less susceptible to synaptopathy than are males, suggesting that sex hormone provide protection. Second, we have shown effective protection against noise-induced synaptopathy with systemic IEM-1460 – injected intraperitoneally – in males and females, possibly making intracochlear injection via surgery unnecessary. Third, we have shown that the GluA2 remains associated with postsynaptic densities (PSDs) during excitotoxic trauma in vitro suggesting that the trauma does not itself increase CP-AMPA. Interestingly, the effect of noise exposure on GluA2 localization in vivo differs between males and females.					
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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. Such noise-induced “synaptopathy” can result in tinnitus and poor speech comprehension in a noisy background, a very 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 previously identified the route of Ca^{2+} entry as via Ca^{2+} -permeable AMPA-type glutamate receptors (CP-AMPA receptors). Using physiological measures – auditory brainstem response (ABR) threshold and amplitude – and direct counting of synapses in confocal microscope images, we have shown that intracochlear perfusion of IEM-1460, a blocker of CP-AMPA receptors, is highly effective in protecting cochlear synapses from noise-induced synaptopathy and thereby preventing the consequent hearing impairment. We now extend this result to show that IEM-1460 injected systemically (intraperitoneally in this case) is likewise effective in preventing noise-induced synaptopathy – this is far more practical therapeutically than intracochlear perfusion and is a major advance. We also show that female mice are less susceptible than are males to noise-induced synaptopathy and differ in response of glutamate receptors to noise. Finally, we have begun to investigate use of the neurotrophic factor CNTF in promoting synapse regeneration.

KEYWORDS

Anandamide
Auditory Brainstem Response
Calcium Ion
Calcium-Permeable AMPA Receptors
Cochlea
Excitotoxicity
Sex Differences
Glutamate Agonist
Glutamate Receptor
Hair Cell
Hearing Threshold
Noise-Induced Hearing Loss
Organotypic Culture
Spiral Ganglion Neuron
Synapse
Synaptopathy

ACCOMPLISHMENTS:

Major goals of the project (from SoW) – approximate % completion to date in *italics*

Major Task 1: Assess protective effect of IEM-1460 delivered by intracochlear perfusion. *100% completed*

Major Task 2: Assess the efficacy of round window delivery of IEM-1460 to prevent noise damage to synapses in vivo. *100% completed*

Major Task 3: Assess the efficacy of systemic delivery of IEM-1460 to prevent noise damage to synapses in vivo. *~50% completed*

Major Task 4: Immunohistochemical determination of intracellular location of GluA2. *~80% completed*

Major Task 5: Assessment of the ability of IEM-1460 with an osmoprotectant to prevent excitotoxic damage to cochlear synapses in vitro. *100% completed*

Major Task 6: Physiological assessments of synapse function in vitro. *0% completed*

Major Task 7: Assess the ability of intracochlear delivery of mannitol or mannitol with IEM-1460 to prevent noise damage to synapses in vivo. *100% completed*

Major Task 8: Assess the ability of systemic delivery (intravenous injection) of mannitol or mannitol with IEM-1460 to prevent noise damage to synapses in vivo. *100% completed*

Major Task 9: Assess the ability of protective treatments to promote regeneration of synapses post-noise by administering the agents after the noise exposure. *~10% completed*

Major activities

- Quantitative analysis of protective effect of IEM-1460 in vitro
- Quantitative analysis of protective effect of IEM-1460 delivered by intracochlear perfusion
- Quantitative analysis of protective effect of IEM-1460 delivered systemically
- Quantitative analysis of effect of gender on susceptibility to synaptopathy
- Assessment of GluA2-PSD95 colocalization in vivo
- Assessment of GluA2-PSD95 colocalization in vitro
- Quantitative analysis of effects of neurotrophic factors on synapse regeneration in vitro

Specific objectives for the reporting period

- a) Complete the in vitro dose response studies for IEM-1460
- b) Complete studies of protection by intracochlearly-perfused IEM-1460 against noise-induced synaptopathy in vivo.
- c) Determine whether there is a sex difference in susceptibility to noise-induced synaptopathy? Are females or males more susceptible?
- d) Assess prevention of synaptopathy with IEM-1460 injected intraperitoneally immediately prior to noise exposure (as opposed to intracochlear infusion, which requires surgical intervention). Is alternative round window delivery necessary or practical?
- e) Quantitation of GluA2 colocalization with other synaptic components: (i) is GluA2 preferentially lost from synapses during noise? (ii) Is there a sex difference in colocalization of synaptic components? (iii) is GluA2 preferentially lost from synapses during excitotoxic trauma (kainic acid) in vitro?
- f) Does CNTF promote synapse regeneration in vitro as does NT-3?

Significant Results (in order corresponding to specific objectives)**a) Quantitative analysis of protective effect of IEM-1460 in vitro**

Major Task 5: Assessment of the ability of IEM-1460 with an osmoprotectant to prevent excitotoxic damage to cochlear synapses in vitro.

Methodology:

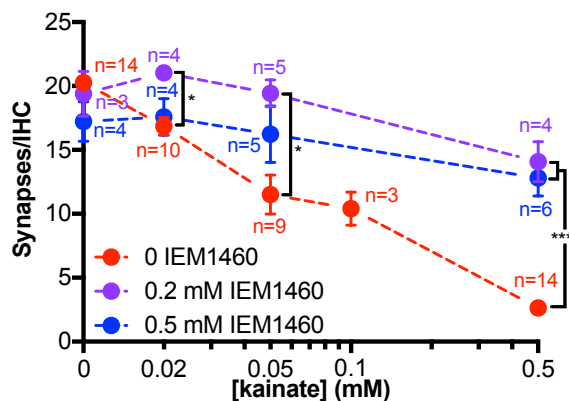
Using neonatal (postnatal day 5, P5) rat cochleae, a portion of the organ of Corti and corresponding part of the spiral ganglion – these experiments use the middle of the cochlea – is transferred to a culture dish where it can be maintained for days. The organotypic explant culture maintains organ of 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 kainic acid (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.

Major findings:

We have established in prior reports that noise exposure (100 dB SPL x 2 hr) causes a reduction in the number of synapses of 25%-30% and an intracochlear perfusion of 0.5 mM IEM-1460 is completely protective against noise-induced synaptopathy. To optimize the *in vitro* model of synaptopathy, we titrated the KA concentration to identify a concentration that causes a synapse loss similar to that caused by moderate noise *in vivo*. This allows rapid *in vitro* screening of protective strategies or regenerative strategies that can then be tested *in vivo*. The data shown in Fig. 1 indicate that 0.02-0.05 mM KA causes the same 25%-30% synapse loss as that caused by moderate noise *in vivo*. This has allowed us to show that IEM-1460 effectively prevents synapse loss due to glutamate excitotoxicity. The data show *complete protection* from KA excitotoxicity at 0.02 mM, 0.05 mM, and significant protection at 0.5 mM.

Figure 1. Number of synapses surviving after a 2 hr exposure to the indicated concentrations of kainate and the indicated concentrations of IEM-1460. Shown are means \pm SEM for indicated number (n) of cochleae. Significance of differences among conditions for each value of [kainate] were determined by ANOVA with posthoc Tukey's multiple comparisons test.

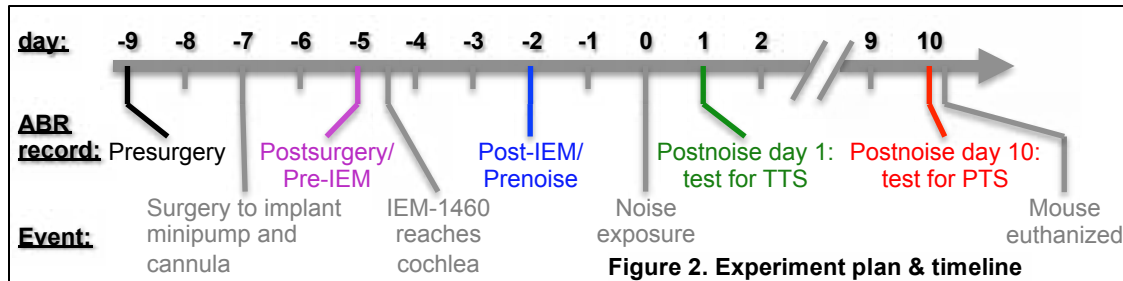


Major Task 5 originally proposed to determine whether addition of an osmoprotectant would potentiate the protective effect of IEM-1460. However, we have shown that IEM-1460 alone provides complete protection at concentrations of KA that replicate the degree of synapse loss due to noise *in vivo* so addition of another potentially protective agent could not improve protection. Moreover, we previously reported that the osmoprotectant mannitol actually caused synapse damage so was counterproductive.

b) Quantitative analysis of protective effect of IEM-1460 delivered by intracochlear perfusion

Major Task 1: Assessment of protective effect of IEM-1460 delivered by intracochlear perfusion.

Major Task 7: Assess the ability of intracochlear delivery of mannitol or mannitol with IEM-1460 to prevent noise damage to synapses *in vivo*.

Methodology:

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 μ l/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 (\approx 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**). This further verifies that IEM-1460 does not affect normal hearing nor reduce sensitivity to noise. If the noise exposure has only damaged synapses and not killed hair cells, then we would expect no permanent threshold shift (PTS); that is, threshold would return to the prenoise level after recovery. In contrast, ABR amplitude would not recover if synapses were damaged and would remain low, especially evident at higher sound pressure levels. If IEM-1460 protects synapses then we would expect the ABR amplitude to return to prenoise levels. We allow 10-14 days for recovery then test ABR: **postnoise day 10**.

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.).

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. As we have previously reported, this noise level generally causes a TTS of 35-40 dB but no PTS. 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 1/4" 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 filter 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% NaN₃ 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.

Major findings:

The characteristics of synaptopathy are (1) no permanent threshold shift (PTS) but a permanent reduction in ABR wave I amplitude and (2) a reduced number of afferent synapses on inner hair cells. In previous reports (not repeated here) we have shown that ABR thresholds reliably recover by 10-14 days after noise exposure. 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 regardless of whether the ears are unoperated, intracochlearly perfused with artificial perilymph (AP) or intracochlearly perfused with IEM-1460 in AP, indicating that *neither the surgery nor the IEM-1460 affects sensitivity of the cochlea to noise*.

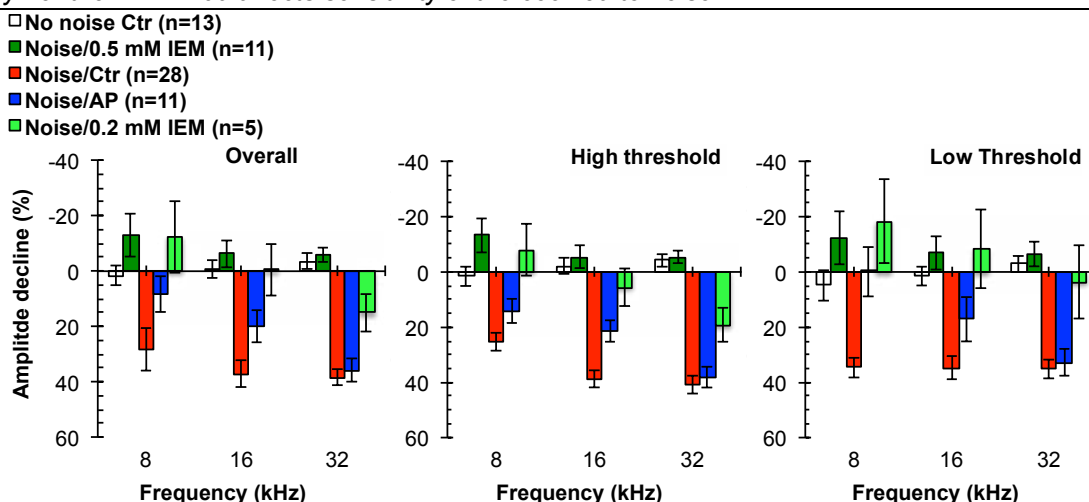


Figure 3. % Decline in ABR wave I amplitude fourteen days after a 2 hr exposure to 100 dB SPL 8-16 kHz octave band noise. Shown are means \pm SEM.

- **No noise Ctr:** control mice not exposed to noise.
- **Noise/Ctr:** 14 days post-noise, unoperated ear
- **Noise/0.5 mM IEM:** 14 days post-noise, intracochlear 0.5 mM IEM-1460 during noise
- **Noise/0.2 mM IEM:** 14 days post-noise, intracochlear 0.2 mM IEM-1460 during noise
- **Noise/AP:** 14 days post-noise, intracochlear vehicle (artificial perilymph) only during noise

Detailed statistics for Figure 3:

Overall. One-way ANOVA for comparisons of ABR amplitude decline among different experimental groups at 8, 16 and 32 kHz respectively are: $F(4, 63) = 14.36, 31.46$ and 60.5 , and $P < 0.0001$ for all.

- Holm-Sidak corrected multiple comparison test to Noise/AP control: significantly different to all groups at 8, 16, 32 kHz, except not significantly different to Noise/Ctr at 32 kHz, to No noise Ctr and to Noise 0.2 mM IEM at 8 kHz
- Holm-Sidak corrected multiple comparison test to No noise control: not significantly different to 0.5 mM and 0.2 mM IEM groups at 8, 16, 32 kHz, and also not significant different to AP at 8 kHz, but significantly different to 0.2 mM IEM at 32 kHz.

Low threshold. One-way ANOVA for comparisons of ABR amplitude decline among different experimental groups at 8, 16 and 32 kHz respectively are: $F(4, 63) = 16.13, 13.6$ and 27.6 , and $P < 0.0001$ for all.

- Holm-Sidak corrected multiple comparison test to Noise/AP control: significantly different to 0.5 mM and 0.2 mM IEM-1460 at 16, 32 kHz, but not at 8 kHz, and also not significantly different to No noise control at 32 kHz.
- Holm-Sidak corrected multiple comparison test to No noise control: not significantly different to 0.5 mM and 0.2 mM IEM-1460 groups at 8, 16, 32 kHz, and also not to No noise control at 8, 16 kHz

High threshold. One-way ANOVA for comparisons of ABR amplitude decline among different experimental groups at 8, 16 and 32 kHz respectively are: $F(4, 63) = 16.5, 37.86$, and 48.7 , and $P < 0.0001$ for all.

- Holm-Sidak corrected multiple comparison test to Noise/AP control: significantly different to all groups at 8, 16, 32 kHz, except not significantly different to No noise control at 32 kHz.
- Holm-Sidak corrected multiple comparison test to No noise control: not significantly different to 0.5 mM and 0.2 mM IEM-1460 groups at 8, 16, 32 kHz, but significantly different to 0.2 mM IEM-1460 group at 32 kHz.

ABR amplitude: We have now completed analysis of all ABR wave I amplitude measurements at 8, 16, and 32 kHz. These data are summarized in Figure 3. Rather than show all of the growth curves, we summarized the % decline between the prenoise amplitude and the amplitude 14 days postnoise. The first panel (Overall) shows the % decline averaged over *all* stimulus levels used (see Figure 3 for example) to generate the growth curve. We also separated out the amplitude decline for the high threshold part of the growth curve (≥ 35 dB SPL) and the low threshold part of the growth curve (< 35 dB SPL.) This is because published reports from the Liberman lab have suggested that high threshold synapses are more susceptible to synaptopathy. Although we do see comparable decline in wave I amplitude (30%-40%) in low and high threshold region of the growth curve, we find that 0.2 mM IEM-1460 does not protect the high threshold response at 32 kHz as effectively as it protects the low threshold response. However, at lower frequencies, 8 and 16 kHz, 0.2 mM IEM-1460 appears completely effective in preventing the noise-induced decline in amplitude. 0.5 mM IEM-1460 is completely effective in preventing the noise-induced decline in amplitude for high- and low-threshold regions at all frequencies tested.

Synapse number: We have now completed quantitation of synapses for all experimental groups at all frequencies and the results are shown in Figure 4. The results of the synapse counts are consistent with the results of the ABR wave I amplitude measures. Synapse loss is completely prevented by 0.5 mM IEM-1460 at all cochlear locations. Synapse loss is prevented by 0.2 mM IEM-1460 at the 8 kHz and 16 kHz locations but only partially prevented synapse loss at the 32 kHz location. Artificial perilymph alone has no significant protective effect. *We have therefore completed Major Task 1 – a milestone establishing IEM-1460 as an effective protectant for synapses against noise damage.* We additionally proposed to compare synaptopathy between males and female mice. All previous studies have used only males and the data of figures 3 and 4 are from males only. To extend studies of the protective effect of IEM-1460 to females, we quantified noise-induced synaptopathy in females (section c, immediately following) and have assessed the protective effects of systemically delivered IEM-1460 in both male and female mice (section d).

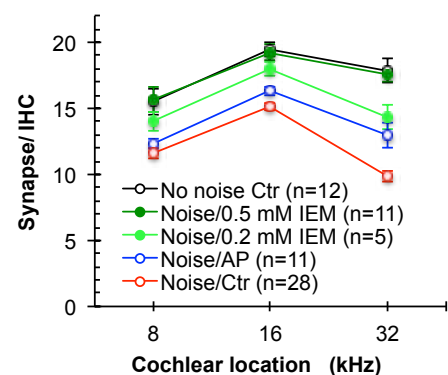
These data indicate that, as was the case for IEM-1460 treatment in vitro, IEM-1460 treatment in vivo appears to be completely protective making it impossible to observe any additional protection by mannitol and obviating the need for use of the osmoprotectant.

Figure 4. Number of synapses (means \pm SEM) surviving after noise exposure in vivo for the indicated number of cochleae at three cochlear locations, 8, 16, and 32 kHz. Cochleae are either **No noise Ctr** not exposed to noise, noise-exposed unoperated (**Noise/Ctr**), noise-exposed perfused with **0.5 mM IEM-1460** or **0.2 mM IEM-1460** or with control artificial perilymph (**Noise/AP**).

Detailed statistics for Figure 4:

One-way ANOVA for comparisons of synapse counts different experimental groups at 8, 16 and 32 kHz regions respectively are: $F(4,63) = 18.19, 36.27, 13.6$, and 47.43 , and $P < 0.0001$ for all.

- Holm-Sidak corrected multiple comparison test to Noise/AP control: significantly different to 0.5 mM at 8, 16 and 32 kHz and 0.2 mM IEM-1460 at 16 kHz, but not at 8 kHz and 32 kHz; also not to No noise control at 8 kHz.
- Holm-Sidak corrected multiple comparison test to No noise control: not significantly different to 0.5 mM and 0.2 mM IEM-1460 groups at 8, 16, 32 kHz, except for significant to 0.2 mM IEM-1460 at 32 kHz, and to Noise/Ctr and No noise control at 8, 16 and 32 kHz. All other differences between groups are significant: (adjusted $p < 0.0001$ to < 0.01)



c) Quantitative analysis of effect of gender on susceptibility to synaptopathy

Major Task 1: Assessment of protective effect of IEM-1460 delivered by intracochlear perfusion.

The experiments above, largely performed in the first year of this project and completed in the present reporting period, used male mice to definitively show a protective effect of IEM-1460 in vivo. This has been the case in all previous studies of synaptopathy, performed in several laboratories. In this project, we proposed to include female mice in our study to determine whether sex hormones affect susceptibility to synaptopathy or the effectiveness of preventive therapies. We initiated these studies in the present

reporting period. We can report our preliminary results that we indeed find sex differences in that *females appear to be less susceptible than males to noise-induced synaptopathy*.

ABR wave I amplitude: Mice were exposed to noise as described above. Figure 5 compares male and female growth curves for prenoise and 14 days postnoise. These show the typical decline in wave I amplitude persisting 14 days postnoise in spite of lack of PTS. However, amplitude decline is significantly greater in males than in females.

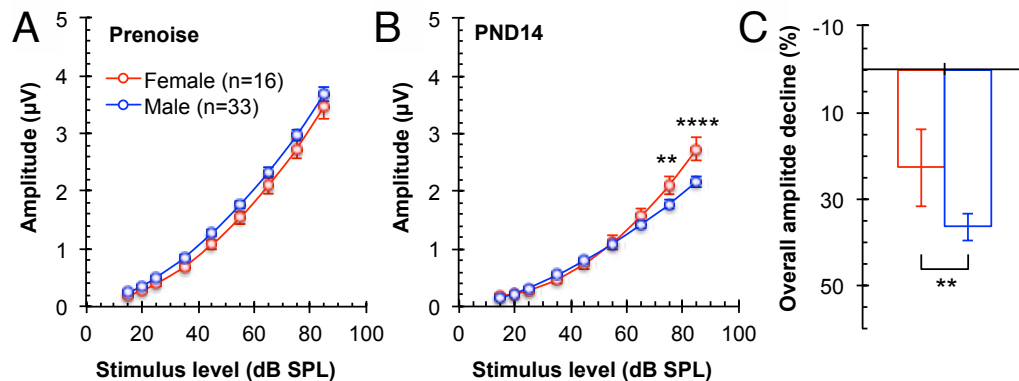


Figure 5. ABR wave I amplitude measurements for 16 kHz tone pips at indicated sound levels. Shown are means \pm SEM. The curves were constructed by fitting the data (by least squares) to a second-order polynomial. These compare prenoise (**A**) and postnoise day 14 (**PND14**, **B**) growth curves from male and female mice. Overall amplitude decline over the entire growth curve is shown in **C**, with amplitude decline significantly greater in males. Significance of differences determined by t-test: * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$

Synapse counts: A similar conclusion can be reached by direct synapse counts (Figure 6). These show that synapse number is significantly reduced in both male and female mice post-noise. However, synapse loss is significantly greater in males than in females.

Figure 6. Synapse counts at the 16 kHz location in control and postnoise day 14 (PND14) male and female mice.

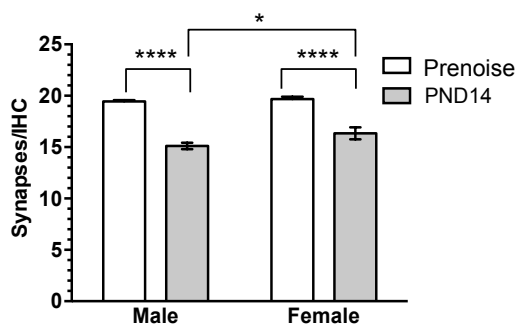
Statistics: Two-way ANOVA for synapse counts between male vs. female and control vs. PND14 groups:

M vs. F for D0 & N14: $F(1, 61) = 4.076$, $p = 0.0479$

D0 vs. N14 for M & F: $F(1, 61) = 90.93$, $p < 0.0001$

Turkey's multiple comparison tests:

**** $p < 0.0001$, * $p < 0.01$



d) Quantitative analysis of the protective effect of IEM-1460 delivered systemically

Major Task 2: Assess the efficacy of round window delivery of IEM-1460 to prevent noise damage to synapses in vivo.

Major Task 3: Assess the efficacy of systemic delivery of IEM-1460 to prevent noise damage to synapses in vivo.

Major Task 8: Assess the ability of systemic delivery (intravenous injection) of mannitol or mannitol with IEM-1460 to prevent noise damage to synapses in vivo.

The major objective of this project is to determine whether IEM-1460 can be used as a protectant in individuals exposed to noise. If so, intracochlear injection is hardly an ideal means of administration. We have therefore initiated efforts to determine whether systemic injection of IEM-1460 immediately prior to noise can be protective. While the data presented here are preliminary, there has been much progress since the previous quarterly report and, with increasing numbers of male and female mice, much of the uncertainty has been cleared and the results now show significant protection by systemic injection.

We originally proposed to approach systemic administration gradually, in three steps. First, we would show that intracochlear perfusion of IEM-1460 – a very invasive approach – is protective against noise-

induced synaptopathy, then we would show that round window delivery, a somewhat less invasive approach though one still requiring surgery under anesthesia. Only after these two approaches were successful would we try a nonsurgical systemic delivery, simply injecting IEM-1460 into the mice. The reason for beginning with intracochlear perfusion is that, had we begun with systemic delivery and obtained negative results, we would not know whether the results were negative because IEM-1460 was not protective or because it did not gain access to the cochlea. Showing first that IEM-1460 is protective when directly introduced into the cochlea means that, if we get a negative result with systemic delivery, the reason would be failure of the compound to gain access to the cochlea, not that the compound is ineffective, and future research could be directed at the problem of access.

Round window delivery was proposed as just such an alternative approach to access. However, the data now show that round window delivery is not a viable approach and that this task is essentially completed. First, as will be evident in our recently obtained data, the systemic approach has shown positive results. Second, our experiments using round window delivery have had negative results and unexpected technical problems. The means we proposed for round window delivery was to load the IEM-1460 into gelfoam or alternative carrier and apply it to the round window. However, we find that delivery by this means is effective for only about two days. Because we allow at least two days for recovery from the surgery before noise exposure, this means that the IEM-1460 supply is depleted prior to the noise exposure. These results argue that the round window approach is not viable and we have focused on systemic delivery via intraperitoneal injection.

Methodology: Noise exposure, ABR, histology, data acquisition and analysis are as described above for mice used for intracochlear perfusion experiments. The key differences are that for systemic injection there is no surgery; rather, IEM-1460 in saline is injected intraperitoneally into mice (12 mg/Kg) 1 hr prior to noise exposure.

ABR results: We first asked whether acute systemic administration of IEM-1460 would affect the wave I threshold or growth curve. As shown in Figure 7 for a representative mouse, there are no detectable changes immediately following IEM-1460 administration. (Note that noise exposure is not a part of this experiment.)

We next measured ABR changes following noise exposure. Here we obtained some very interesting results (Figure 8). Figures 8A and 8B show that for both male and female mice injected with IEM-1460, there is a complete or almost complete recovery of ABR wave I amplitude by 14 days postnoise (PND14). This is in marked contrast to mice exposed to identical noise but not injected with IEM-1460 (Figure 8C). This recovery can be seen by comparing the difference in the prenoise (blue) and PND14 (red) growth curves in panels A and B to the difference between these growth curves in C. The data, while still preliminary, demonstrate a significant protective effect of IEM-1460 injected immediately prior to noise exposure.

Unexpectedly and remarkably, there is an effect of IEM-1460 on the temporary threshold shift (TTS), specifically, a significant reduction in the degree of magnitude of the TTS, i.e., protection against this temporary effect of noise. This can be seen by the steeper slope of the postnoise day 1 growth curves (green) in Figure 8 A and B, relative to PND1 growth curve in C. At high stimulus levels the response amplitude is near the prenoise level. It is clear from our studies and those of other laboratories that synaptopathy has a negligible and undetectable effect on threshold. Even if systemic IEM-1460 protects against synaptopathy, it should, nevertheless, have no protective effect against TTS, which is due to damage to outer hair cells, not to synapses. Indeed, as we have shown, *intracochlear* IEM-1460 has no effect on TTS so the relevant target must be outside of the cochlea but there is no obvious target consistent with the data. While we do not yet understand this effect, it is evidently beneficial effect and does not argue against the use of IEM-1460 to protect against noise.

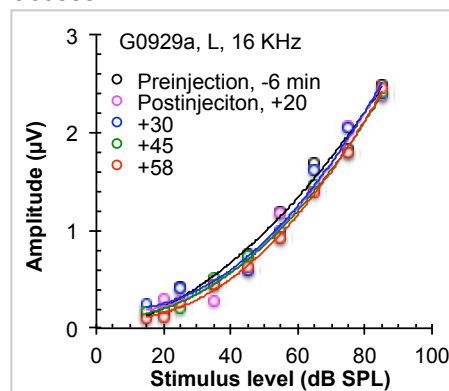


Figure 7. ABR wave I amplitude measurements for 16 kHz tone pips at indicated sound levels. Shown are means \pm SEM. The curves were constructed by fitting the data (by least squares) to a second-order polynomial. These compare preinjection (6 min before injection) and ABR measurements at the indicated times (in minutes) postinjection.

These data indicate that, as was the case for IEM-1460 treatment in vitro, IEM-1460 treatment in vivo appears to be completely protective making it impossible to observe any additional protection by mannitol and obviating the need for use of the osmoprotectant.

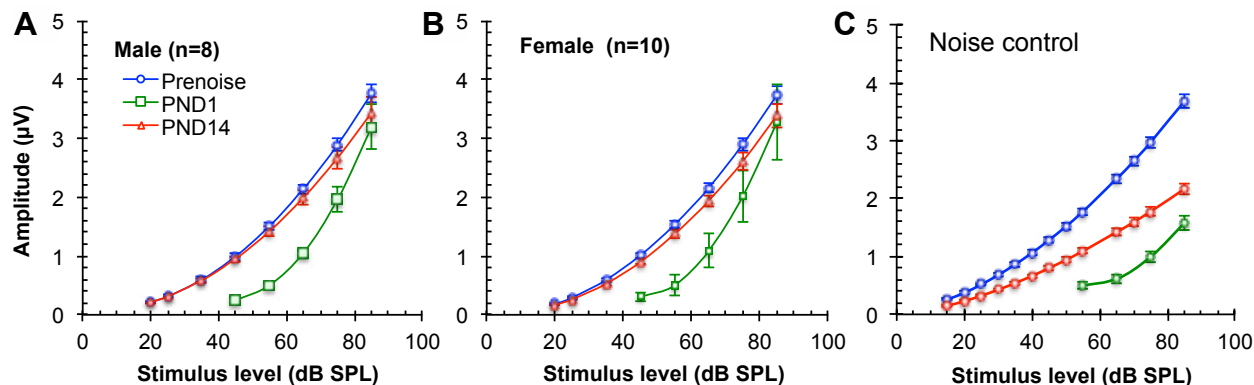


Figure 8. ABR wave I amplitude measurements for 16 kHz tone pips at indicated sound levels at each of the following timepoints: **Prenoise**, postnoise day 1 (**PND1**) to show TTS and acute effects of noise on amplitude growth, and postnoise day 14 (**PND14**) to show PTS and permanent effects of noise on amplitude growth. Shown are means \pm SEM. **A** and **B** show growth curves from, respectively, male and female mice injected intraperitoneally with 12 mg/Kg IEM-1460 prior to noise exposure; **C** shows growth curves from 23 male control mice not treated with IEM-1460 for purpose of comparison to show protection by IEM-1460.

e) Quantitation of GluA2 colocalization with other synaptic components

Major Task 4: Immunohistochemical determination of intracellular location of GluA2.

Our observation that a blocker of Ca^{2+} -permeable AMPA receptors (CP-AMPA) 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-AMPA receptors 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-AMPA receptors on the surface;
- (3) GluA2 is always present at all synapses but in the population of glutamate receptors at each synapse, many or most of the receptor tetramers contain only GluA3 and/or GluA4 subunits but lack a GluA2 subunit. These would therefore be Ca^{2+} -permeable.

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.

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 synaptopathic noise in vivo or to KA in vitro.

(e)(i) is GluA2 preferentially lost from synapses in vivo during noise?

Methodology

Mice were exposed to noise and immunolabeled as on p. 7 (Histology and Imaging) but with the inclusion of anti-GluA2 antibodies. Figure 9 shows examples.

Significant Results

In this reporting period we have *increased the number of replicates and compared the consequences of noise between male and female mice*. Representative images are shown in Figure 9 and quantified data are in Figure 10. The data show sex differences in colocalization of GluA2 and PSD95 immediately

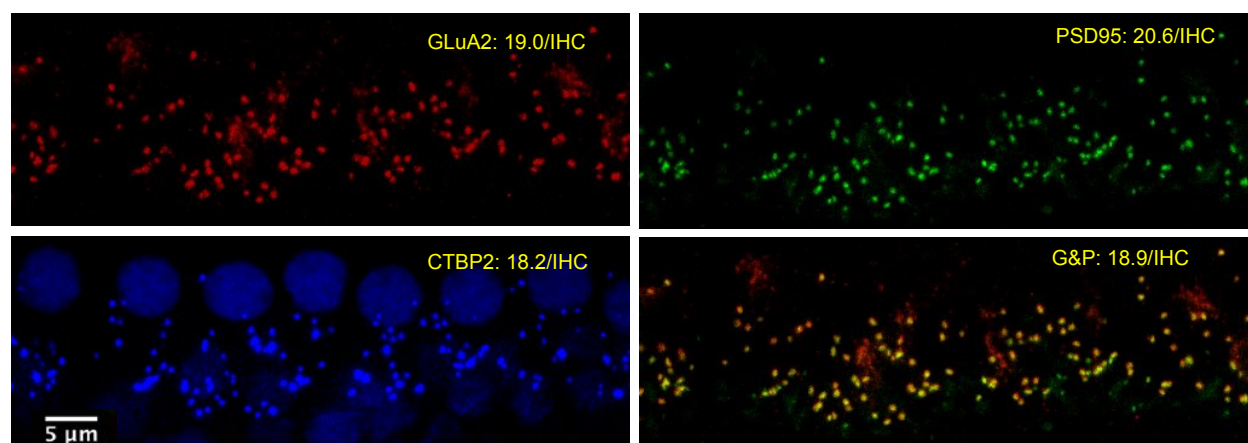


Figure 9. Labeling of GluA2 (G, red), PSD95 (P, green) and CtBP2 (C, blue) in a cochlea immediately after a 2 hr noise exposure. The number of immunolabeled puncta is indicated in each panel. The panel labeled G&P shows colocalized GluA2 and PSD95 puncta. Note that the number of colocalizations is very close to the number of PSDs implying that after the noise exposure GluA2 remains present at each individual synapse.

postnoise in that males appear to be more susceptible to noise. In comparing PSD95-GluA2 colocalization (P & G) immediately after noise (PND0) these preliminary results show a *significant decline in males but not in females*. This is consistent with the observation that males are more susceptible than females to noise-induced synaptopathy.

(e)(ii) is GluA2 preferentially lost from synapses during excitotoxic trauma (kainic acid) in vitro?

Methodology

Cultures were prepared and exposed to various concentrations of KA, as summarized above on p. 3. The cultures were fixed after various exposure times up to 2 hr in KA. Antibodies to GluA2 and, in this report, also to GluA3 were included in the set of antibodies used.

Significant Results

In this report we have added additional replicates to the GluA2-PSD95 measures and consider this part of the study to be completed. We have also begun studies of colocalization of GluA3 with PSD95. While GluA2 is present only in glutamate receptor clusters that are Ca^{2+} -impermeable, GluA3 is present in all glutamate receptor clusters. It will be instructive to monitor colocalization of glutamate receptors with PSD95 generally.

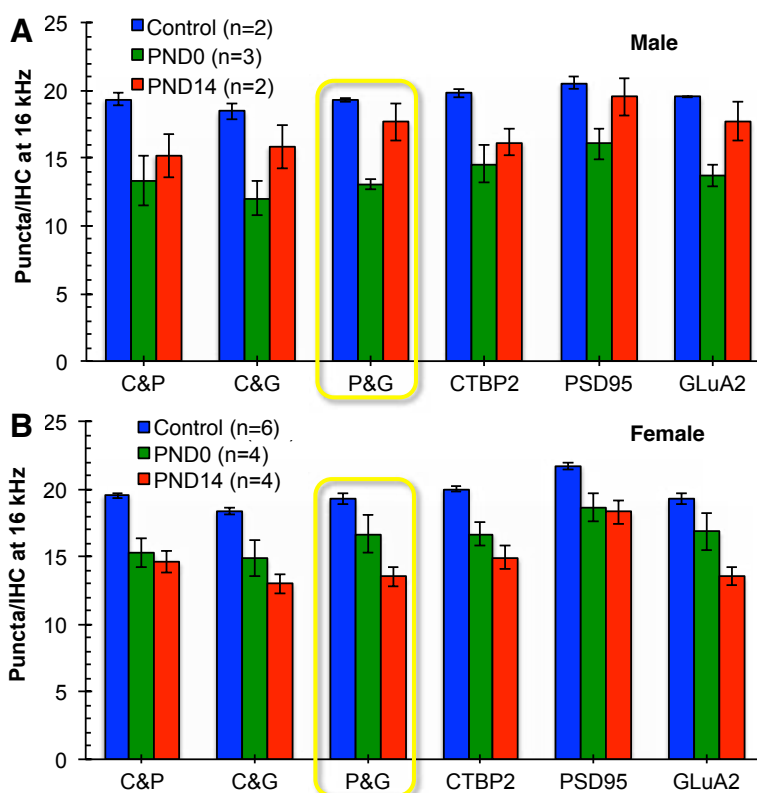


Figure 10. Number (means \pm SEM) of immunoreactive puncta in inner hair cells at the 16 kHz location. **A** males, **B** females. **Control** (blue): control cochleae, not exposed to noise. **PND0** (green): noise-exposed cochleae immediately after noise exposure. **PND14** (red): noise-exposed cochleae 14 days after noise exposure. Presynaptic ribbons (CtBP2), postsynaptic densities (PSD95), and GluA2 clusters were immunolabeled as in Figure 11 and the numbers of immunoreactive puncta counted and shown in the figure. Also shown are the number of colocalized CtBP2 with PSD95 (C&P,) CtBP2 with GluA2 (C&G,) and PSD95 with GluA2 (P&G.) The latter indicates the number of synapses containing GluA2, the key result, and is highlighted in yellow.

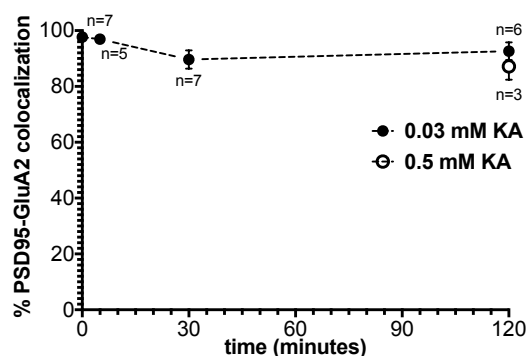


Figure 11. Colocalized GluA2 and PSD95 puncta – i.e., postsynaptic densities containing GluA2 – were counted in the indicated number of cultured cochlea exposed to 0.03 mM KA or 0.5 mM KA for the indicated time intervals. The %age of synapses (PSD95-immunoreactive puncta) containing GluA2 immunoreactivity was calculated and is plotted here. Shown are means \pm SEM.

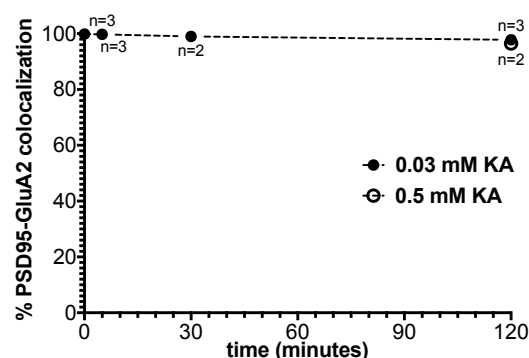


Figure 12. Colocalized GluA3 and PSD95 puncta – i.e., postsynaptic densities containing GluA3 – were counted in the indicated number of cultured cochlea exposed to 0.03 mM KA or 0.5 mM KA for the indicated time intervals. The %age of synapses (PSD95-immunoreactive puncta) containing GluA3 immunoreactivity was calculated and is plotted here. Shown are means \pm SEM.

Data shown (Figure 11) are largely from cultures exposed to 0.03 mM KA. We focused on this concentration because this mimics the degree of synapse loss, 5 or 30 min in KA. The percentage of synapses containing GluA2 is not significantly lower during KA exposure than initially. A small apparent reduction is not statistically significant nor can it account for the ~25% reduction in synapses that occurs with 0.03 mM KA. To further confirm this conclusion, we also exposed cultures to 0.5 mM KA that results in loss of nearly all synapses within two hours (Figure 1). Nevertheless, in 87% of the surviving postsynaptic densities GluA2 remained present.

Similar results were obtained from preliminary observations of GluA3 (Figure 12.) Again, nearly all postsynaptic densities retained GluA3 after up to two hours of exposure to 0.03 mM KA or even to 0.5 mM KA.

As noted in the previous progress report we are partnering with the Developmental Studies Hybridoma Bank to develop antibodies to GluA3 and GluA4 that will be better than existing commercial antibodies for immunofluorescence at high resolution. We have completed immunizing mice and are currently testing sera. We have completed development of a screening strategy. Briefly, we obtained expression vectors for GluA2, GluA3, and GluA4, transfected them into COS7 cells to obtain expression of these subunits in the cells and will use this to screen monoclonals for antibodies that can be used for immunofluorescence studies.

(f) Quantitative analysis of effects of neurotrophic factors on synapse regeneration in vitro.

Major Task 9: Assess the ability of protective treatments to promote regeneration of synapses post-noise by administering the agents after the noise exposure.

To accomplish this aim, we initiated in vitro studies of the abilities of various neurotrophic factors to promote synapse regeneration in vitro. This will allow us to identify the most effective factors. In the next reporting period we will initiate studies of the ability of the most promising factor to promote synapse regeneration in vivo after noise-induced synaptopathy.

Our previous studies have shown that NT-3 is capable of promoting synapse regeneration in vitro. Recently, work in Gabriel Corfas' lab has confirmed this for synapse regeneration in vivo. However, work in Robin Davis' lab has also shown that NT-3 also changes the physiological properties of spiral ganglion neurons so may not be an ideal means of promoting synapse regeneration. We have published studies showing that ciliary neurotrophic factor (CNTF) is expressed in the organ of Corti at high levels, comparable to NT-3. We have also found that CNTF is approximately as effective as NT-3 in promoting spiral ganglion neuron survival and neurite outgrowth. We therefore compared the ability of CNTF and NT-3 to promote synapse regeneration.

Methodology. Cochlear explant cultures were prepared and exposed to 0.5 mM KA as described on p. 3. However, instead of fixing the cultures within hours after KA exposure, the cultures were incubated for

three days post-KA with 50 ng/mL NT-3, or 50 ng/mL CNTF, or combined CNTF and NT-3 or no neurotrophic factor (NTF.) The cultures were then fixed, labeled, and imaged as described on p. 3.

Significant Results. The completed study is shown in Figure 13. These data show that CNTF is promising as a neurotrophic factor for synapse regeneration should NT-3 prove inappropriate. However, CNTF is slightly but significantly less effective than NT-3 in promoting synapse regeneration.

Training: A new graduate student Sepand Bafti 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 39th Annual MidWinter Meeting, Baltimore, MD, Feb 2016. A paper is being submitted on IEM-1460.

Plans for next reporting period. In the next reporting period (Oct 2016 – Oct 2017) we plan to focus on the following main objectives:

- Our highest priority is completing a quantitative assessment of the efficacy of systemic delivery (injection) of IEM-1460 immediately prior to noise exposure in preventing synaptopathy and comparing its effectiveness in male and female mice (Major Task 3).
- Our second priority is assessment of the ability of neurotrophic factors – especially CNTF – to promote synapse regeneration in male and female mice when administered subsequent to noise exposure. Time permitting, we will investigate other agents that can potentially be used for this purpose (Major Task 9).
- We consider work on use of our in vitro model to assess the efficacy of IEM-1460 to be essentially complete. We plan in vitro experiments only in limited cases to screen particular compounds for efficacy in promoting synapse regeneration prior to testing in vivo (Major Task 9) and to assess the integrity of regenerated synapses (Major Task 6).
- 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. Work in the last reporting period focused on the in vitro model. Work in the next reporting period will focus on noise-induced changes in vivo in male and female mice (Major Task 4). Also, to this end, we will complete development of new antibodies to detect glutamate receptor subunits.

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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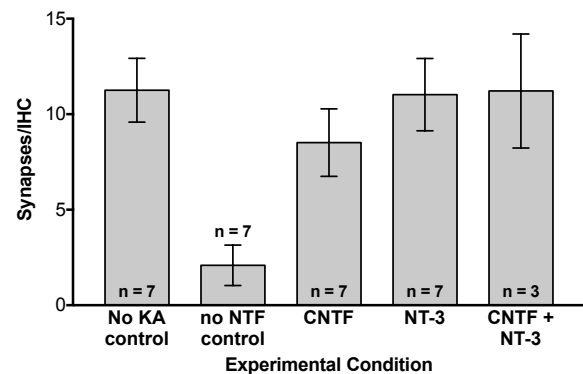


Figure 13. Number of synapses present after a 2 hr exposure to 0.5 mM KA or no KA control followed by three days incubation with the indicated neurotrophic factor or no NTF control. Shown are means \pm SD for indicated number (n) of repetitions.

- One-way ANOVA for comparisons of synapse counts: $F = 48$, and $P < 0.0001$ for all.
- Tukey's multiple comparison test: **No KA control, CNTF, NT-3, CNTF+NT-3** are all significantly different from **no NTF control**, $p < 0.001$. **CNTF** is significantly different from **NT-3, CNTF+NT-3**, and **no KA control**, $p < 0.05$. Other differences are not significant.

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: Results from this and other laboratories provide compelling evidence that even moderate noise can cause permanent hearing impairment. This is a serious societal problem affecting individuals in noisy environments and those with whom they must interact. Military personnel and veterans are especially at risk. The research presented here shows promise in development of pharmacological protective and therapeutic strategy. Nevertheless, these can be viewed as “last resorts” and society should be concerned about appropriate protective strategies through noise abatement, ear protection and changes in regulation and workplace environments.

Changes in approach: There are no significant changes to report.

Changes affecting expenditure: We have three significant changes to report:

Sriram Hemachandran, a graduate student working on the project received a fellowship from our partner, the Developmental Studies Hybridoma Bank to develop antibodies to detect glutamate receptor subunits (see p. 14). We designated Mr. Hemachandran's stipend to support another graduate student (Mr. Bafti) to work on this project. However, delays in recruiting the student mean that funds could not be fully spent in the previous reporting period and will be spent instead in the next.

Dr. Sivan-Loukianova was hired to work on this project and completed studies shown in Figures 11 and 12. However, she decided to retire and we have not yet been able to hire a replacement, which has resulted in unspent funds from the previous reporting period that will be spent in the next reporting period after we can recruit a suitable replacement.

Continued use of in vitro experiments to screen compounds resulted in a decrease in the number of animals we had expected to use in the previous reporting period temporarily reducing costs for our rat and mouse colonies. However, emphasis on in vivo experiments in the next reporting period will result in a significant increase in our animal costs and use of the unexpended funds.

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:	Elena Sivan-Loukianova
Project Role:	Research Scientist
Nearest person month worked:	4
Contribution to Project:	Planning experiments; Microscope imaging and analysis of digital images; culture of organotypic cochlear explants

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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: 2
Contribution to Project: Microscope imaging and analysis of digital images; culture of organotypic cochlear explants and spiral ganglion neurons. Development of monoclonal antibodies.

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

Special Reporting Requirements: The quad chart is appended.

There is no appendix.