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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. *~80% completed*

Major Task 4: Immunohistochemical determination of intracellular location of GluA2. *~95% 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. *essentially completed as an in vivo experiment.*

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. *~70% completed*

Major activities

- Quantitative analysis of protective effect of IEM-1460 delivered systemically
- Quantitative analysis of effect of gender on susceptibility to synaptopathy
- Quantitative analysis of effect of steroid sex hormones on susceptibility to excitotoxic trauma in vitro
- Assessment of GluA2-PSD95 colocalization in vivo
- Quantitative analysis of effects of neurotrophic factors on synapse regeneration in vitro
- Quantitative analysis of effects of neurotrophic factors on synapse regeneration in vivo

Specific objectives for the reporting period *match letters to sections in Significant Results*

- a) Quantitative analysis of the protective effect of IEM-1460 delivered systemically.
- b) Quantitative analysis of effect of gender on susceptibility to synaptopathy.
- c) Quantitation of GluA2 colocalization with other synaptic components: is GluA2 preferentially lost from synapses in vivo during noise?
- d) Quantitative analysis of effects of neurotrophic factors on synapse regeneration in vitro.
- e) Quantitative analysis of effects of neurotrophic factors on synapse regeneration in vivo.

Significant Results

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

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

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 showed 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*. The major objective of this project is to determine whether IEM-1460 can be used as a protectant in individuals exposed to noise. Data presented previously show that IEM-1460 delivered intracochlearly in mice effectively prevents noise-

induced cochlear synaptopathy (NICS). However intracochlear injection is hardly an ideal means of administration for protection in humans. 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, the results 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 NICS, 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 in previous reports 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.

Auditory Brainstem Response (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:

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 1 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.)

ABR changes following noise exposure (Figure 2). As previously reported, and 2B show that for both male (Figure 2A) and female (Figure 2B) mice injected with 12 mg/Kg 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 2C). 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. These data demonstrate a significant protective effect of IEM-1460 injected immediately prior to noise exposure.

These data indicate that, as was the case for IEM-1460 treatment in vitro or intracochlearly, it is also the

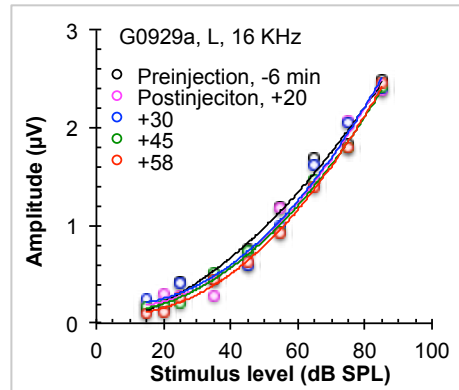


Figure 1. ABR wave I amplitude measurements for 16 kHz tone pips at indicated sound levels. Shown are means ± SEM. The curves were constructed by fitting the data (by least squares) to a second-order polynomial. These compare preinjection (6 min before injection) and ABR measurements at the indicated times (in minutes) postinjection.

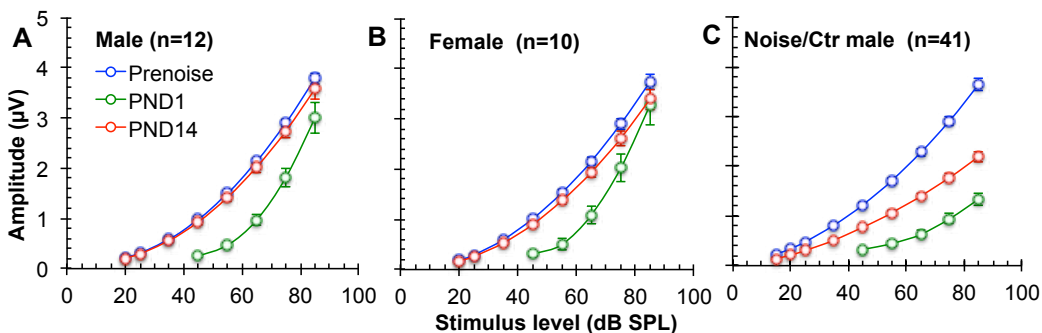


Figure 2. ABR wave I amplitude measurements for 16 kHz tone pips at indicated sound levels at each of the following timepoints: **Prenoise**, postnoise day 1 (**PND1**) to show TTS and acute effects of noise on amplitude growth, and postnoise day 14 (**PND14**) to show PTS and permanent effects of noise on amplitude growth. Shown are means ± 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 male control mice not treated with IEM-1460 for purpose of comparison to show prevention of amplitude decline by IEM-1460.

case that systemic treatment with IEM-1460 in vivo appears to be strongly protective, making it practically impossible to observe any additional protection by mannitol as we had originally proposed and obviating the need for use of the osmoprotectant.

Figure 3 compares normalized ABR wave I amplitudes between control and systemically IEM-1460-treated male and female mice. These data show that in IEM-1460-treated males the overall decline in wave I amplitude at PND14 is only 2.9%; significantly ($p < 0.0001$) less than that in untreated control males. In female mice, IEM-1460 was less effective at protection and the decline in wave I amplitude was not significantly different than in control mice. While the lack of significant difference is due, in part, to a smaller effect of IEM-1460 in females than in males, a major contributing factor to the lack of significance is a smaller effect of noise on wave I amplitude, i.e., a smaller degree of synaptopathy in females. Also, in females, there is greater variability among individuals in the degree of synaptopathy (see below). These data demonstrate a significant difference between males and females in NICS, which we are further investigating (see below).

Reduced concentration of IEM-1460: To minimize any possible off-target (“side”) effects of IEM-1460, we have initiated studies to determine the lowest effective concentration. Specifically we reduced the IEM-1460 concentration by three-fold (~1/2 log unit) to 4 mg/Kg. *This experiment is novel to this progress reporting period, not an extension of previously reported experiments, but the results should still be considered preliminary.* To control for any effects of the injection itself on sensitivity to noise (for example, the stress of the injection could affect the mouse’s response) we included an additional control condition to the experiment, specifically a vehicle-only injection. (The IEM-1460 vehicle is saline.)

As shown in Figure 4, 4 mg/Kg IEM-1460 is effective in reducing the effect of noise on ABR wave I amplitude. However, the effect may be only partial, the amplitude does appear to be reduced from the prenoise level. The vehicle injection had no effect in either increasing or decreasing the effect of noise on ABR wave I amplitude. This experiment used only males. As will be discussed below in detail, for females, susceptibility varies with phase of the estrous cycle. To do this experiment with females would therefore require four times the number of males to insure a comparable number of females in every one of the four stages of the mouse estrous cycle. This was not known at the time the grant application was written and the budget developed and is not possible with the funds and time remaining. We have submitted grant applications to continue study of these issues in female mice. Having said that, we point out that IEM-1460 was significantly less effective in females even at 12 mg/Kg so would likely have only marginal effectiveness in females at a concentration of 4 mg/Kg.

Data from all of the preceding experiments is summarized, for purpose of comparison and statistical analysis in Figure 5. These data show that 4 mg/Kg and 12 mg/Kg systemic IEM-1460 both significantly reduce the noise-induced hearing impairment. With the smaller number of subjects receiving 4 mg/Kg, it’s not possible to detect a significant difference between 4 and 12 mg/Kg although it appears that 4 mg/Kg is partially effective and 12 mg/Kg entirely effective and likely to be the preferred dose.

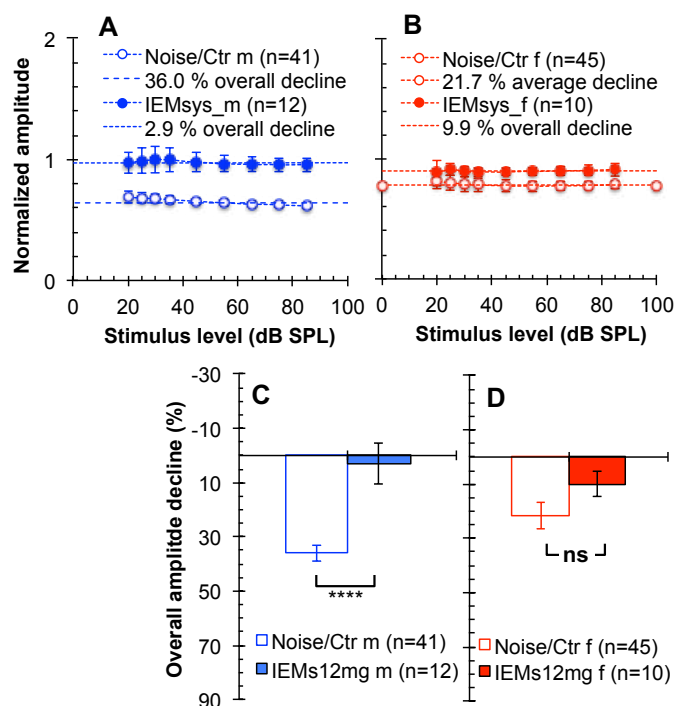


Figure 3. Normalized ABR wave I amplitude measurements for 16 kHz tone pips at indicated sound levels. Each amplitude measure at PND14 was normalized to the prenoise measure for the same mouse at that amplitude. The plots compare **Control** mice to mice receiving IEM-1460 systemically (**IEMsys**). Panel **A** shows the results for male mice; panel **B** shows the results for female mice. Overall differences between control and IEM-1460-treated mice, averaged over all stimulus levels, are shown in panels **C** (male mice) and **D** (female mice). The difference between control and IEMsys in overall amplitude decline is significant (t-test, $p < 0.0001$) for males but not for females.

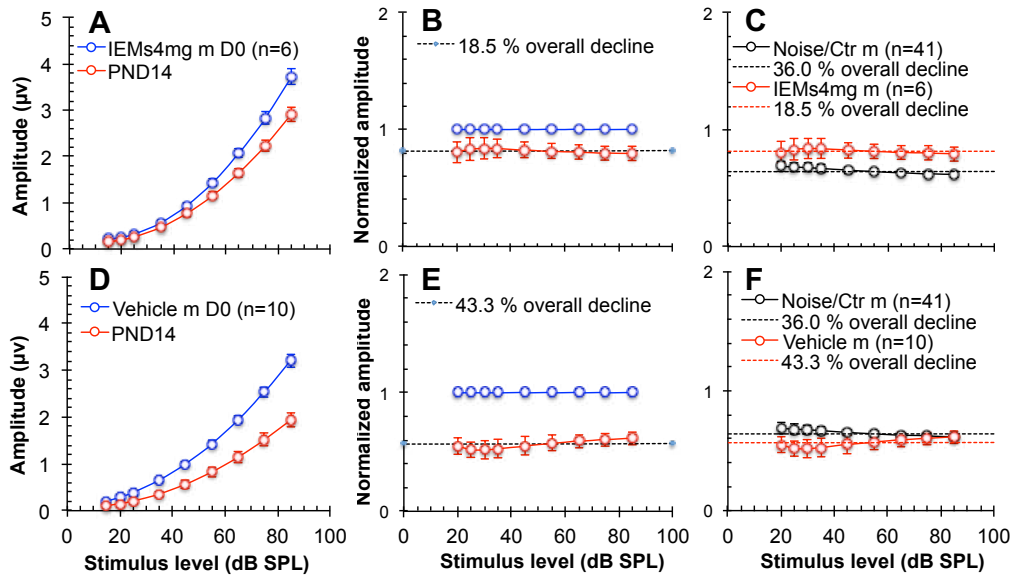


Figure 4. Comparison of prenoise to postnoise day 14 (PND14) ABR wave I amplitude measurements for 16 kHz tone pips at the indicated sound levels. A-C injection of 4 mg/Kg IEM-1460 in saline; D-F injection of vehicle only. A,D amplitude measurements. B,E amplitude measures normalized to prenoise measures for each mouse. C,F direct comparison of normalized amplitude measures with those from noise-exposed uninjected control mice. Note that overall decline for vehicle-injected mice is similar to that of uninjected mice while overall amplitude decline for mice injected with 4 mg/Kg IEM-1460 is significantly smaller, indicating protection by 4 mg/Kg IEM-1460.

Results from synapse counts: Counting synapses (Figure 6) yields results similar to, but more promising than, those from ABR measurements. In this reporting period, the numbers of Noise/Ctr males and females are increased and the number of IEMsys males has been increased. These data confirm that females are less susceptible to NICS than are males (see below) but show a highly significant and essentially complete protective effect of systemic IEM-1460. Synapse number shows no significant reduction after noise damage in mice treated with systemic IEM-1460 (@ 12 mg/Kg), in contrast to the loss of synapses observed in control noise-exposed mice. These new data further confirm that vehicle alone has no effect, neither positive nor negative, and that 4 mg/Kg IEM-1460 is protective against NICS.

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

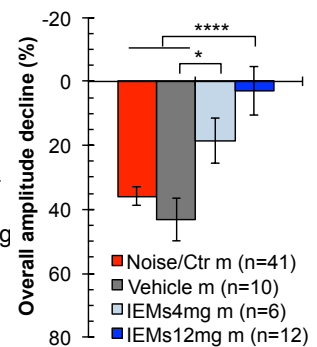
Major Tasks 1 & 3: Assessment of protective effect of IEM-1460 in male and female mice.

The data shown in Figures 3 and 6 imply that female mice are less susceptible than males to NICS. Here we more precisely quantify the difference.

ABR wave I amplitude: Mice were exposed to noise as described above. Figure 7 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.

Synapse counts: Direct synapse counts (Figure 8) show that synapse number is significantly reduced in both male and female mice post-noise. Synapse loss appears significantly although only slightly greater in males than in females. This is in spite of the much greater reduction in ABR wave I amplitude in males than in females. This suggests that differences between males and females are much more pronounced on the physiological than the structural level.

Figure 5. Comparison of overall amplitude decline among noise-exposed male (m) mice: untreated noise-exposed mice (Noise/Ctr), vehicle injection control (Vehicle), for systemic IEM-1460 (IEMs) at 12 mg/Kg (12mg) or 4 mg/Kg (4mg). The difference between noise-exposed untreated and vehicle-injected is not significant (ns) as is the difference between mice injected with 12 mg/Kg or 4 mg/Kg IEM-1460. The difference between IEM-1460-treated and untreated or vehicle-injected mice is significant ($p < 0.05$, Kruskal-Wallis with Dunn's multiple comparison test).



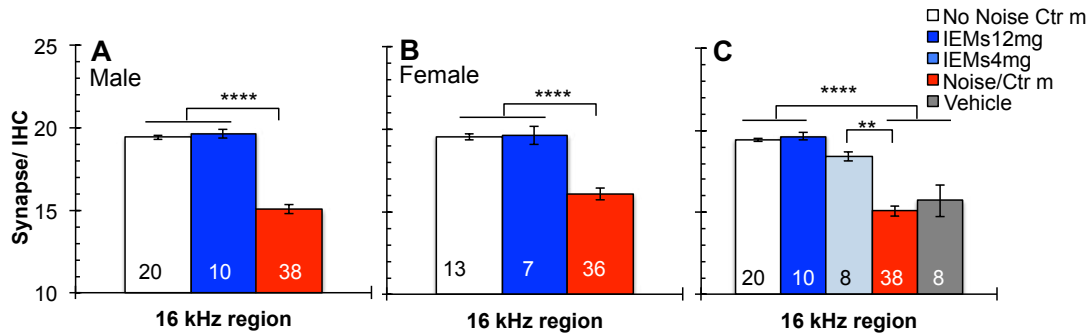


Figure 6. Synapse counts at the 16 kHz region in male (A) and female (B) cochleae from non-noise exposed control (No Noise Ctr, white) mice, control noise-exposed (Noise/Ctr, red) mice and noise-exposed mice systemically treated with 12 mg/Kg IEM-1460 (IEMs12mg, blue). There is no significant difference in synapse number between No Noise and IEMs12mg mice but synapse number in noise/Ctr mice was significantly (ANOVA, $p < 0.0001$) smaller. C. Comparison (for male mice only) of synapse counts among all experimental conditions, those in A as well as saline/vehicle and systemic 4 mg/Kg IEM-1460 (IEMs4mg). There is no significant (ns) difference between synapse counts in noise-exposed untreated and vehicle-injected mice and no significant difference among synapse counts in IEM-1460-treated and non-noise exposed mice nor between mice injected with 12 mg/Kg or 4 mg/Kg IEM-1460. The difference between IEM-1460-treated and untreated or vehicle-injected mice is significant ($p < 0.001$, Kruskal-Wallis with Dunn's multiple comparisons test) indicating a protective effect of IEM-1460 against NICS.

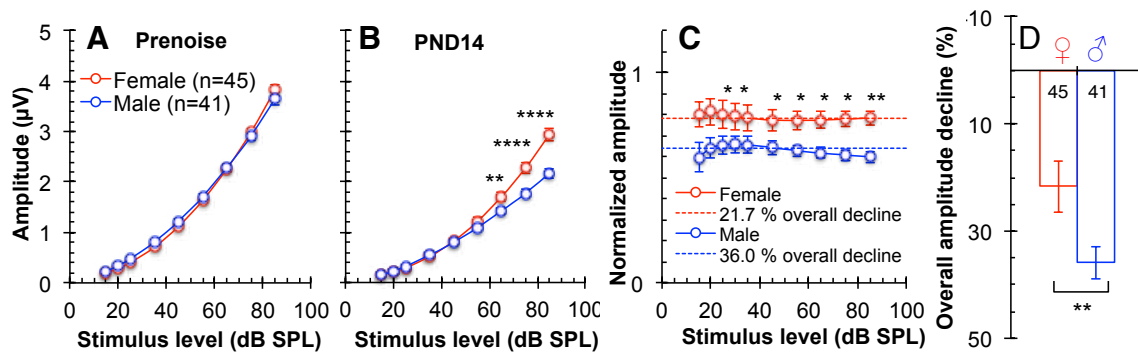


Figure 7. ABR wave I amplitudes for 16 kHz tone pips at indicated sound levels. Shown are means \pm SEM. The curves were constructed by fitting the data (by least squares) to a second-order polynomial. These compare prenoise (A) and postnoise day 14 (PND14, B) growth curves from male and female mice. Normalized ABR wave I amplitude measurements for 16 kHz tone pips at indicated sound levels are shown in C (each amplitude measure at PND14 was normalized to the prenoise measure for the same mouse at that amplitude) for male and female mice. Overall amplitude decline over the entire growth curve is shown in D for male and female mice. Males have a significantly greater amplitude decline. Significance of differences determined by t-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Testosterone is not the reason that male mice exhibit greater susceptibility to synaptopathy. To determine whether males are more susceptible because of male hormones, we compared susceptibility to NICS between control male mice and castrated male mice. As shown in Figure 9, postnatal day 14 wave I amplitude growth curves are substantially similar in male control vs. castrated mice. Direct quantitative comparison (Figure 10) shows no significant difference

We therefore conclude that it is more likely that female hormones are protecting against susceptibility as opposed to male hormones increasing susceptibility.

Correlation of noise-induced cochlear synaptopathy with female sex hormones: The data in Figure 7 show that not only are female mice less susceptible to synaptopathy than are males but also that there is a much greater variability in susceptibility among the female mice than

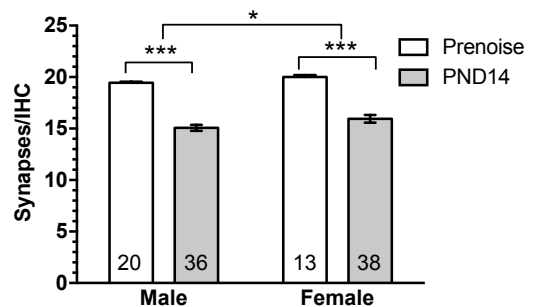


Figure 8. Synapse counts at the 16 kHz location in control and postnoise day 14 (PND14) male and female mice. Differences between control No noise and noise-exposed PND14 are significant for male and female mice). Synapse counts are significantly lower in male noise-exposed mice than in female noise-exposed mice (2-way ANOVA (* $p < 0.05$, *** $p < 0.001$).

among male. Having concluded that it is female sex hormones that are determining susceptibility, we hypothesized that this variability is due to the females being at different points in their estrous cycles and therefore being exposed to noise with different levels of circulating sex hormones present.

To test this hypothesis, we initiated studies to correlate estrous cycle stage at time of noise exposure with the degree of synaptopathy. While, ultimately, we will correlate synaptopathy with hormone levels assayed in blood drawn from female mice at the time of noise exposure, we carried out a preliminary test by using vaginal smears from female mice at the time of noise exposure, labeling with cresyl violet, and using the histological appearance of the cells obtained to determine the estrous phase for each female (McLean et al. Performing vaginal lavage, crystal violet staining, and vaginal cytological evaluation for mouse estrus cycle staging identification. *J Vis Exp* 67:e4389, 2012). The relationship between appearance of cells in the vaginal smear, estrous stage, and hormone level is shown in Figure 12.

Estrous stage, determined from the smears, was correlated with the two measures of synaptopathy for these noise-exposed female mice: reduction in wave I amplitude (Figure 13) and loss of synapses (Figure 14). *In this reporting period we have increased the number of female mice assessed and increased the statistical significance of the result.*

As shown in Figure 13, NICS was significantly less severe for noise exposure during the diestrous stage than for other stages as determined by ABR wave I amplitude. Alignment with hormone levels shows that this is the stage with elevated progesterone, raising the possibility that progesterone is directly or indirectly protective against NICS. This is an intriguing possibility given that progesterone has been observed to be neuroprotective in some neurodegenerative diseases.

As shown in Figure 14, NICS was significantly less severe for noise exposure during the diestrous/high progesterone phase also if reduced synapse number is used as the criterion for NICS. Synapse loss is significantly less than for noise exposure during the proestrous or estrous phases. In fact, synapse number in mice exposed to noise during the diestrous phase is not significantly different

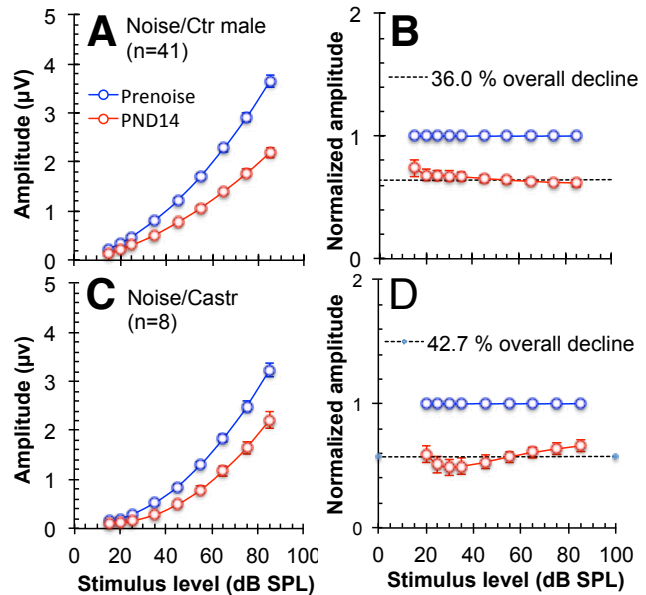


Figure 9. ABR wave I amplitudes for 16 kHz tone pips at indicated sound levels. Shown are means \pm SEM. The curves were constructed by fitting the data (by least squares) to a second-order polynomial. These compare prenoise and postnoise day 14 (PND14) wave I amplitude growth curves from control male (A) and castrated (B) mice. Normalized (as in Figure 3) ABR wave I amplitude measurements for 16 kHz tone pips at indicated sound levels are shown for control males in C and for castrated males in D.

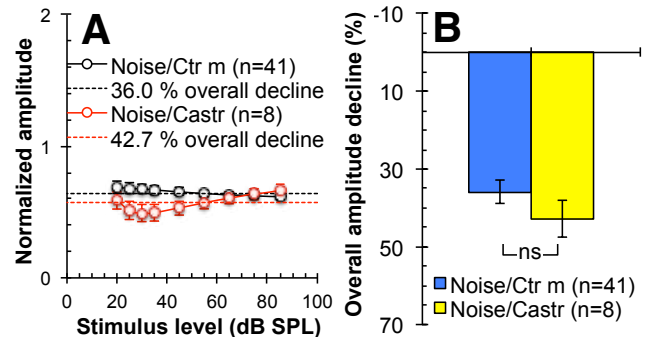
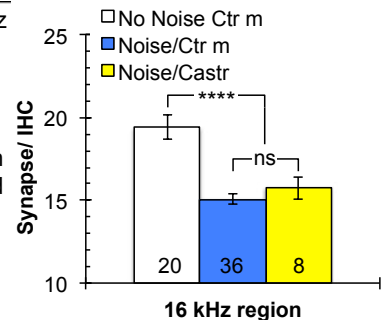


Figure 10. Overall amplitude decline over the entire stimulus intensity range (from Figure 9 B & D) are shown in A for noise-exposed control male (Noise/Ctr) and castrated (Noise/Castr) mice. These overall amplitude declines are compared in B. There is no significant difference (t-test) between control and castrated males.

Figure 11. Synapse counts at the 16 kHz region in noise-exposed control male (Noise/Ctr) and castrated (Noise/Castr) mice compared to control male mice not exposed to noise (No Noise Ctr) mice. The number of subjects is shown in each column. Noise-exposed male control and castrated mice have significantly fewer synapses than non-noise exposed control males but are not different from each other (ANOVA, Holm-Sidak post-hoc test, $p < 0.0001$).



than synapse number in female mice not exposed to noise. These data argue for a strong protective effect of progesterone. We consider this to be a very important and valuable result.

What is the mechanism by which progesterone maintains synapses in noise-exposed mice?

We have begun to investigate this question by considering two (non-mutually exclusive) alternatives. One is that the presence of progesterone during noise exposure protects the synapses and prevents their being lost in the first place. The second is that synapses are lost but progesterone promotes a rapid regeneration of the synapses. Following our previously established strategy, we have begun by investigating *in vitro* using our organotypic culture system in which experiments can be conducted more quickly and will subsequently follow up with experiments on noise-exposed mice.

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 KA, generally at 0.5 mM, for 2 hr (same time duration as the noise exposure *in vivo*.)

To test the ability of agents to provide protection, they are included in the culture medium with the KA. In these experiments, progesterone was added at 20 ng/ml, which is approximately the peak concentration of progesterone during the diestrous phase in mice. The culture is then fixed and synapses counted to determine the extent of synapse loss with or without the putative protective agent(s). The same

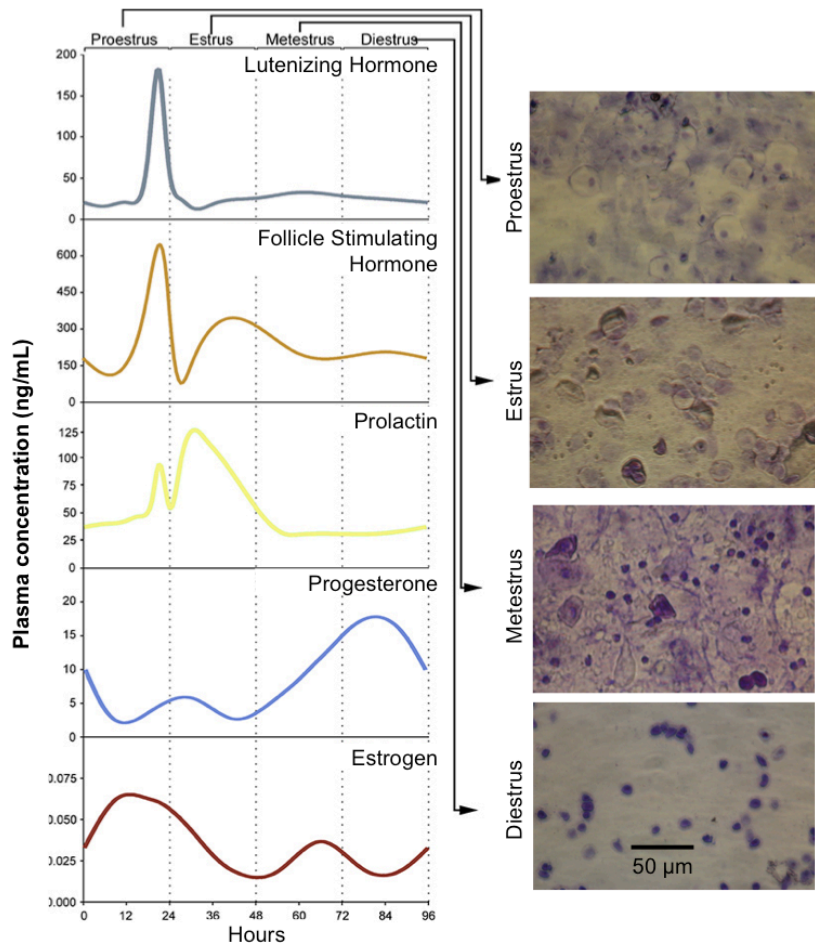
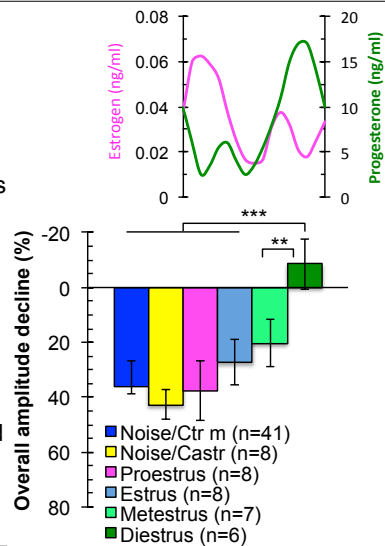


Figure 12. Relationship between appearance of cells in a vaginal smear, estrus stage, and hormone level, was modified from McLean et al. (2012) by substitution of vaginal smear images done in our lab for those of McLean et al. to demonstrate our ability to carry out this analysis.

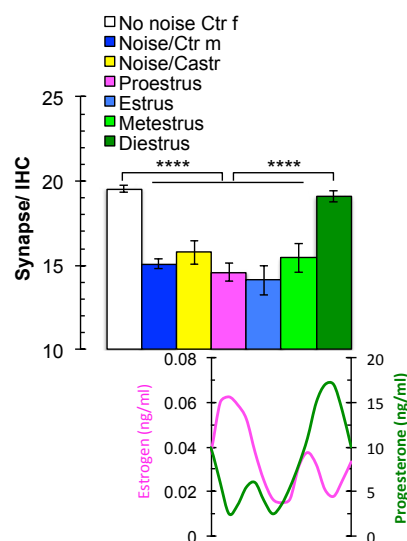
Figure 13. Sensitivity to NICS appears to vary with phase in the estrous cycle: ABR wave I amplitude measures: ABR wave I amplitude (16 kHz tone pips) decline (as defined in Figure 3) in females at different estrous stages compared to the mean amplitude decline in all noise-exposed females (**Noise/Ctr**) previously tested without regard to estrous stage. Shown are means \pm SEM. There is no significant difference (ns) between amplitude decline in unstaged noise-exposed controls and females in proestrus and estrus stages. However, amplitude decline for noise exposure in the diestrous stage is significantly reduced ($*p < 0.05$, ANOVA, Holm-Sidak correction for multiple comparisons) and is undetectable. The estrous cycle stage results are aligned with graphs of corresponding hormone levels from Figure 12.



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.

Figure 14. Sensitivity to NICS appears to vary with phase in the estrous cycle: synapse counts:

Synapse counts at the 16 kHz location in females at different estrous stages compared to counts in all noise-exposed females (**Noise/Ctr**) previously tested without regard to estrous stage. Shown are means \pm SEM. There is no significant difference (ns) in synapse loss among unstaged noise-exposed controls and females in proestrous and estrous stages. Also, there is no significant difference between synapse loss in these females and synapse loss in noise-exposed castrated males (**Noise/Castr**). However, synapse number in mice exposed to noise during the diestrous stage is significantly greater ($***p < 0.0001$, ANOVA, Holm-Sidak correction for multiple comparisons). Moreover, synapse number in mice exposed to noise during the diestrous stage is not significantly different from synapse number in mice not exposed to noise (**No noise**). The estrous cycle stage results are aligned with graphs of corresponding hormone levels.



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.

To test agents for ability to promote regeneration, the cultures are incubated for three days after the KA exposure to allow time for synapse regeneration. Agents to be tested are included in the culture medium during the three day incubation. In these experiments, progesterone was added at 20 ng/ml, which is approximately the peak concentration of progesterone during the diestrous phase in mice. We also quantified synapse regeneration in cultures with a comparable concentration of β -estradiol (10 ng/ml). Although this is >3 orders of magnitude higher than the *in vivo* concentration, we want to ask whether the reduced susceptibility to NICS during the diestrous phase is due to the high concentration of steroid sex hormone or, specifically, due to progesterone. After three days, the cultures are fixed, labeled with antibodies to detect hair cells, presynaptic ribbons, and PSDs and synapses are counted.

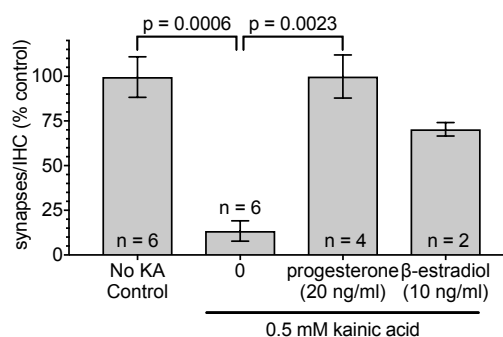


Figure 15. Number of synapses present after a 2 hr exposure to 0.5 mM kainic acid (KA) followed by three days incubation with the indicated steroids. Synapse counts in each experiment were normalized to a No KA control within the experiment. Shown are means \pm SD for indicated (in the column) number of repetitions. Significance of differences was determined by one-way ANOVA with Holm-Sidak multiple comparisons test.

Significant results. We found no significant protective effect of progesterone (data not shown). That is, the presence of progesterone during exposure to 0.5 mM KA did not significantly affect the loss of synapses (typically ~90%) caused by this excitotoxic treatment. In contrast, as shown in Fig. 15, we found that the presence of progesterone during the three days post-KA resulted in significant recovery of synapse number. β -estradiol at high concentration appears to have a similar effect although this is a very preliminary result (n=2) and not statistically significant. These data suggest that the reason that female mice are resistant to NICS during the diestrous phase is because the high concentration of steroid sex hormone promotes a rapid regeneration of damaged synapses. This hypothesis will have to be tested in *in vivo* in future experiments.

c) Quantitation of GluA2 colocalization with other synaptic components: is GluA2 preferentially lost from synapses *in vivo* during noise?

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

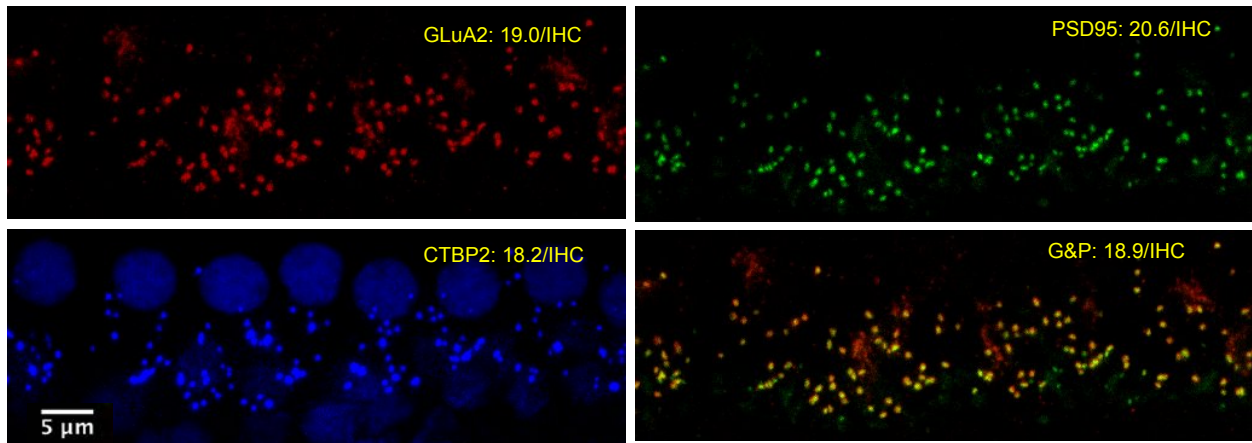


Figure 16. Labeling of GluA2 (G, red), PSD95 (P, green) and CtBP2 (C, blue) in a cochlea immediately after a 2 hr noise exposure. The number of immunolabeled puncta is indicated in each panel. The panel labeled G&P shows colocalized GluA2 and PSD95 puncta. Note that the number of colocalizations is very close to the number of PSDs implying that after the noise exposure GluA2 remains present at each individual synapse.

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.

Methodology

Mice were exposed to noise and immunolabeled as on p. 5 (Histology and Imaging) but with the inclusion of anti-GluA2 antibodies. Figure 16 shows examples.

Significant Results

Representative images are shown in Figure 16 and quantified data are in Figure 17. *In this reporting period we have increased the number of replicates but there are no qualitative changes in our conclusions.* Comparison of data from male and female mice show sex differences in colocalization of GluA2 and PSD95 immediately 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 NICS.

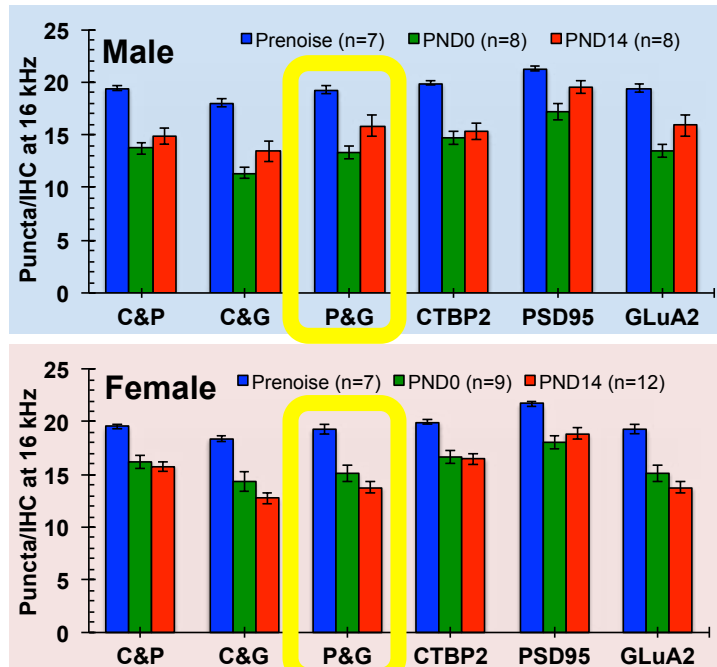


Figure 17. Number (means ± SEM) of immunoreactive puncta in inner hair cells at the 16 kHz location for male (upper) and female (lower) mice.

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 10 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.

d) 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.

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: We used the in vitro protocols described above in section (b) (page 12) for quantitation of synapse regeneration three days after excitotoxic trauma. In these experiments, the three day incubation is with neurotrophic factors (NTFs) to test their ability to promote regeneration: 50 ng/mL NT-3, or 50 ng/mL CNTF, or combined 50 ng/mL CNTF and 50 ng/mL NT-3, or no NTF control.

Significant Results. Kainic acid (kainate, KA) was applied at two different concentrations: 0.03 ng/mL, which causes a loss of ~25% of synapses (Figure 18A), mimicking the effect of noise in vivo, and 0.5 mM, which destroys nearly all synapses (Figure 18B). At 0.03 mM KA, increased synapse regeneration was observed at 50 and 150 ng/mL CNTF, significant at 150 ng/mL CNTF. However, with increased synapse damage, at 0.5 mM KA, Synapse recovery was significant at both 50 ng/mL and 150 ng/mL CNTF, comparable to NT-3 and to combined NT-3 and CNTF. These studies suggest assessment of CNTF as a means of promoting synapse regeneration post-noise in vivo and suggest that 50 ng/mL CNTF is a minimal effective dose. We have initiated these studies.

e) Quantitative analysis of effects of neurotrophic factors on synapse regeneration in vivo.

Methodology:

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

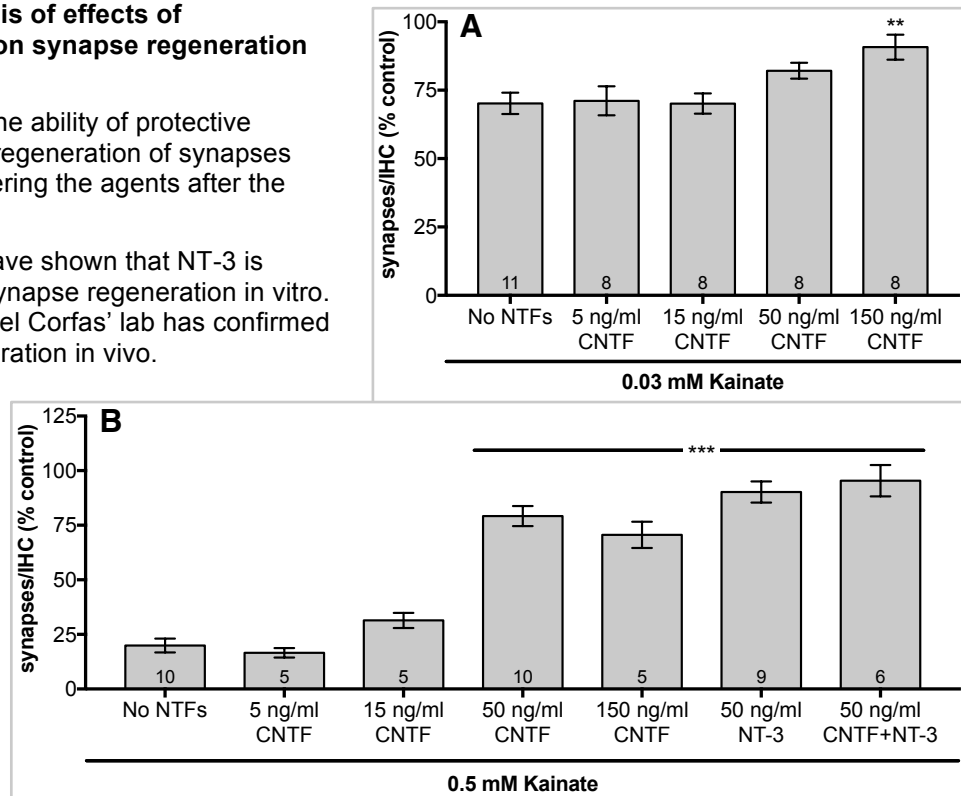


Figure 18. Number of synapses present after a 2 hr exposure to 0.03 mM kainate (A) or 0.5 mM kainate (B) followed by three days incubation with the indicated NTF(s) or no NTF control. Synapse counts in each experiment were normalized to a no kainate control within the experiment. Shown are means ± SD for indicated (in the column) number of repetitions. Significance of difference from **No NTFs** control was determined by one-way ANOVA with Holm-Sidak multiple comparisons test, **p<0.01, ***p<0.001. **A.** Synapse regeneration in response to increasing concentrations of CNTF over 1½ log units at ½ log unit increments after exposure to 0.03 mM KA. **B.** Synapse regeneration in response to increasing concentrations of CNTF over 1½ log units at ½ log unit increments and comparison to NT-3 and combined CNTF and NT-3, after exposure to 0.5 mM KA.

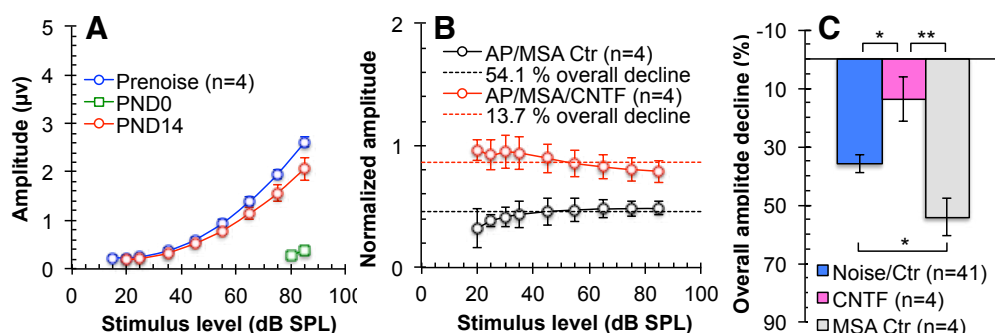


Figure 19. CNTF rescues post-noise wave I amplitude decline in vivo. **A.** ABR wave I amplitude measurements for 16 kHz tone pips at indicated sound levels. Shown are means \pm SEM. The curves were constructed by fitting the data (by least squares) to a second-order polynomial. The graph compares **Prenoise**, immediate postnoise (**PND0**, showing TTS) and postnoise day 14 (**PND14**) growth curves. Note that in CNTF-treated mice, amplitudes have recovered to nearly the prenoise level. **B.** Normalized ABR wave I amplitude measurements for 16 kHz tone pips at indicated sound levels. Each amplitude measure at **PND14** was normalized to the prenoise measure for the same mouse at that amplitude. Compared are ABR amplitude measures from ears receiving CNTF in MSA-containing AP to ears receiving control MSA in AP. **C.** Overall amplitude decline is by \sim 35% for noise-exposed control unoperated mice and even greater, \sim 50%, for mice receiving control AP/MSA but only \sim 15% for mice receiving CNTF in AP/MSA. * $p=0.036$ (MSA Ctr vs. nonoperated Ctr), * $p=0.011$ (CNTF vs. nonoperated Ctr), ** $p=0.0013$, one-way ANOVA, Holm-Sidak multiple comparisons test.

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. 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.).

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 temporary threshold shift (TTS) of 35-40 dB but no permanent threshold shift (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.

Assessment of synapse regeneration: Twelve week old CBA/CaJ mice are exposed to a moderate noise that destroys synapses on inner hair cells (IHCs) but spares the hair cells themselves. Approximately 2 1/2 hrs postnoise, the mice are surgically implanted with a minipump-cannula system providing 100 ng/mL CNTF in artificial perilymph (AP) with 0.1% mouse serum albumin (MSA) or control AP with MSA and no CNTF. The Alzet model 2004 is a 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 is filled with the experimental solution AP/MSA control or AP/MSA with CNTF so that the CNTF will enter the cochlea within \sim 1/2 day after noise exposure and be available for at least two weeks.

The consequences of the noise are assessed by auditory brainstem response (ABR) to assess the degree of synaptopathy physiologically – wave I amplitude – and immunohistochemistry to count synapses directly. ABR is first assessed prior to noise exposure to determine the baseline value, used to normalize postnoise responses. ABR is again measured 30 min – 2 hr post-noise exposure (but prior to the surgery) to assess the temporary threshold shift (TTS), which should be 35-40 dB if the noise has been properly calibrated to cause synaptopathy but not damage hair cells. ABR is measured again 10-14 days postnoise to ensure that there is no permanent threshold shift, which would indicate hair cell

damage, and measure the decline in wave I amplitude, which directly indicates the severity of synaptopathy.

Significant Results. These, as yet preliminary, results are very promising. In mice receiving CNTF postnoise, wave I amplitudes were restored to nearly prenoise levels within 14 days; in contrast, mice receiving vehicle only showed typical severely reduced post-noise wave I amplitudes (Figure 19). Similarly, mice receiving CNF showed nearly complete recovery of synapses in direct counts (Figure 20).

Significance: Noise, even at sound levels too low to kill hair cells, can damage or destroy the synapses between hair cells and nerve cells in the ear. While this does not cause profound deafness – the individual may still retain almost normal sensitivity to sound – it does result in serious hearing impairments. Consequences are tinnitus (“ringing in the ears”) and impaired hearing in a noisy background. This means, for example, that the affected individual has trouble following conversation in a room in which there is some noise or, perhaps, one or two other conversations in the background. This type of hearing impairment is common after middle age, especially in men, and more common after noise exposure. It is far more common than complete deafness due to loss of hair cells. Hearing impairments such as tinnitus or diminished ability to hear or follow a conversation in background noise cause significant communication difficulties with family members, co-workers, and friends and leads to isolation and depression, among other problems. These hearing impairments impact work performance and, in some cases, limits military and post-military career options. In 2009, it was estimated that more than 445,000 veterans were receiving services for hearing loss related to active military service. NICS appears to be a significant contributor to these noise-induced hearing impairments. The work described here is focused on identifying novel therapeutics that prevent NICS or promote recovery.

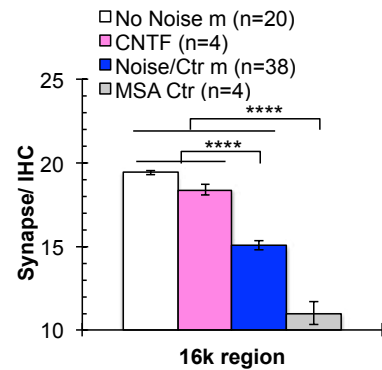


Figure 20. Synapse counts at the 16 kHz location in cochleae from control non-noise exposed (**No Noise**) mice; Noise-exposed unoperated mice (**Noise/Ctr**); control noise-exposed mice receiving intracochlear perfusion of vehicle only (**MSA Ctr**); and noise-exposed mice receiving intracochlear perfusion of vehicle with 50 ng/mL **CNTF**. ****p<0.0001, one-way ANOVA, Holm-Sidak multiple comparisons test.

W81XWH-14-1-0494 Annual Progress Report 29 Oct 2017

Training: two graduate students, Sriram Hemachandran and Sepand Bafti have been trained in Auditory Brainstem Response (ABR) measurements.

Dissemination of Results: These results have been reported at the Association for Research in Otolaryngology 40th Annual MidWinter Meeting, Baltimore, MD, Feb 2017.

Plans for next reporting period. In the next reporting period (Oct 2017 – Oct 2018) 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, in particular, increasing the number and completing analysis of the data we have obtained for 8 kHz and 32 kHz. (Major Task 3).
- A second high priority goal 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).
- A third high priority goal is to increase our confidence, by testing additional female mice, that the diestrus (high progesterone) stage of the murine estrous cycle is a time of minimal susceptibility to noise-induced cochlear synaptopathy. We will also complete the analysis of data we have obtained for 8 and 32 kHz.
- We consider work on use of our in vitro model to assess the efficacy of IEM-1460 to be essentially complete. We plan to use our culture system to determine whether steroid sex hormones have a direct effect on synaptopathy by treating cultures with sex hormones, particularly progesterone, and quantifying synapse loss and synapse regeneration.
- 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. To this end, we expect to develop 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.

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: Nothing 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: 6
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: Mouse surgery; ABR measurement; data analysis; 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: 8
Contribution to Project: ABR measurement; data analysis; microscope imaging and analysis of digital images; culture of organotypic cochlear explants and spiral ganglion neurons. Software development.

Special Reporting Requirements: The quad chart is appended.

There is no appendix.

Prevention of Noise Damage to Cochlear Synapses

W81XWH-14-1-0494 Application MR130438



PI: Steven Green

Org: University of Iowa

Award Amount: \$1,484,000

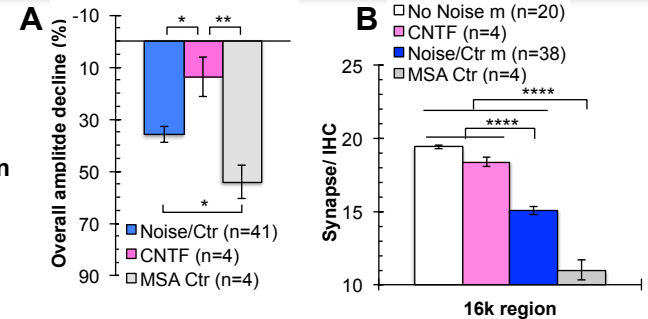
Study Aims

- Does blockade of CP-AMPA receptors in vivo prevent or reduce noise-induced synaptopathy
- Is there a cause of synaptopathy other than Ca²⁺ entry?
- Can neurotrophic stimuli promote synapse regeneration?

Approach

Two means for protection against damage to cochlear synapses will be assessed: (1) IEM1460, a selective blocker of Ca²⁺-permeable AMPA-type glutamate receptors and (2) mannitol, an osmoprotectant. These agents will be initially assessed alone and in combination in vitro for protection against excitotoxicity and in vivo in noise-exposed mice for protection against noise. Neurotrophic stimuli will likewise be tested in vitro and in vivo to determine efficacy in promoting regeneration of synapses already destroyed by excitotoxicity or noise.

Synapse regeneration in vivo promoted by CNTF. A. CNTF rescues post-noise wave I amplitude decline in vivo.



Overall amplitude decline is by ~35% for noise-exposed control unoperated mice and even greater, ~50% for mice receiving control AP/MSA but only ~15% for mice receiving CNTF in AP/MSA. *p=0.036 (MSA Ctr vs. nonoperated Ctr), *p=0.011 (CNTF vs. nonoperated Ctr), **p=0.0013, one-way ANOVA, Holm-Sidak multiple comparisons test. **B. Synapse counts** at the 16 kHz location in cochleae from control non-noise exposed (**No Noise**) mice; Noise-exposed unoperated mice (**Noise/Ctr**); control noise-exposed mice receiving intracochlear perfusion of vehicle only (**MSA Ctr**); and noise-exposed mice receiving intracochlear perfusion of vehicle with 50 ng/mL **CNTF**. ****p<0.0001, one-way ANOVA, Holm-Sidak multiple comparisons test.

Timeline and Cost

Activities	CY	14	15	16
In vitro quantitation of neuroprotection by IEM1460 and/or mannitol vs. kainate		█	█	
Colocalization of postsynaptic densities and glutamate receptors			█	█
Quantitation of protection of synapses vs. noise by intracochlear IEM1460		█	█	
Quantitation of protection of synapses vs. noise by systemic IEM1460			█	█
Quantitation of synapse regeneration vs. noise by neurotrophic stimuli			█	█
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** – Initiate in vivo studies of neuroprotective agents vs. noise
- ☑ Immunohistochemical localization of GluA2 and GluA3 in vitro
 - ☑ Assess protective effect of IEM-1460 delivered by intracochlear perfusion
- CY16 Goal** – Complete in vivo studies of neuroprotective agents vs. noise
- ☐ Immunohistochemical localization of GluA2 and GluA3 in vivo
 - ☐ Assess protective effect of IEM-1460 delivered systemically
 - ☐ Compare noise damage and protection by IEM-1460 between male and female mice and determine hormone(s) relevant to neuroprotection.
 - ☐ Assess synapse regeneration following neurotrophic stimuli delivered by intracochlear perfusion.