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14. ABSTRACT Tinnitus is the perception of sound in the absence of an environmental stimulus. This phantom sound in the head, is most commonly caused by noise exposure, resulting in damage to the inner ear. Within the veteran population seeking VA care, 16-27% suffer from serious hearing loss and tinnitus. Unfortunately, those most affected are bound to the sounds in their heads have difficulty concentrating, suffer from depression and may even contemplate suicide. We posit that breaking the bond between attention and tinnitus will ameliorate the impact of tinnitus. Drugs acting at receptors that bind the brain chemical acetylcholine (nAChRs), a substance involved in brain circuits that control attention could ameliorate tinnitus. We have successfully tested the drug (sazetidine-A), in an established sound-exposure animal model of tinnitus. First year studies focused on understating the role of the nAChRs and in attentional brain circuits. *These 1st year studies obtained data on two questions: 1) How are selective drugs that act at nAChRs involved in circuits that control attention? 2) What tinnitus-related changes occur in the pharmacology and function of nAChRs in the attentional circuits of primary auditory cortex (AI)?*

15. SUBJECT TERMS
Tinnitus, Nicotinic receptor, acetylcholine, auditory cortex, Sazetidine, Varenicline

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Annual Progress Report 7-15-2019 through 7-14-2020: Nicotinic Receptor Pathology in Tinnitus: Auditory Cortex and Selective Desensitizing Nicotinic Agents

1. Introduction

Tinnitus is defined as a phantom sound (ringing in the ears) that can significantly affect the quality of life for those who suffer its effects. The most common cause of tinnitus is excessive noise exposure. Chronic tinnitus is present in 10 to 15 percent of the population, the focus of the present study is on the 5 to 10 percent of those most affected by their tinnitus (Shargorodsky *et al.*, 2010). These sufferers experience depression, anxiety, sleep disturbances, inability to concentrate, fatigue, and occasionally, suicidal ideation (Roberts *et al.*, 2010; Henry *et al.*, 2013; Roberts *et al.*, 2013). The magnitude of their tinnitus distress may relate to having their attention fixed on this phantom auditory percept (Jacobson *et al.*, 1996; Roberts *et al.*, 2013). **Given this dynamic, strategies for treating the attentional aspects of tinnitus may be as important as ameliorating the tinnitus sensation itself.** Studies during the first year focused on basic science and translational treatment strategies to better understand underlying maladaptive attentional mechanisms related to nicotinic cholinergic receptors (nAChRs).

During the first year of *Nicotinic Receptor Pathology in Tinnitus: Auditory Cortex and Selective Desensitizing Nicotinic Agents,* basic science and translational studies sought to understand the role of the nAChRs and novel pharmacological approaches to treating individuals most troubled by their tinnitus. *These studies addressed two questions: 1) How are selective drugs that act at nAChRs involved in circuits that control attention? 2) What tinnitus-related changes occur in the pharmacology and function of nAChRs in the attentional circuits of primary auditory cortex (AI)?*

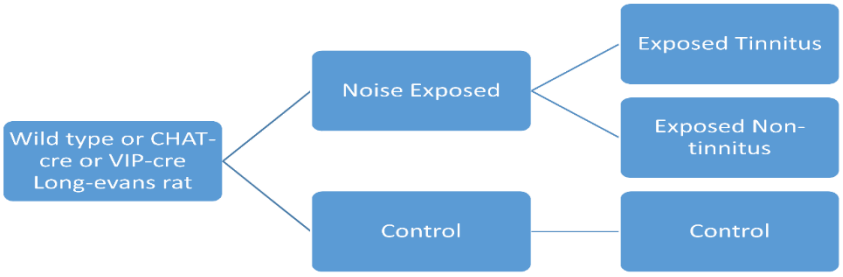

Figure 1. Approach to the generation, assessment and classification of tinnitus animals.

2. Key Words

tinnitus, nicotinic cholinergic receptors (nAChRs), attention, auditory cortex, Sazetidine-A, varenicline

3. Accomplishments (7/15/2019-7/14/2020)

Year#1: During the first year of PR180160, 118 Long-Evans (LE) rats were entered into the study. This included, 52 wild-type LE rats, 62 choline acetyltransferase (ChAT) Cre+ LE rats, 2 vasoactive intestinal peptide (VIP) Cre+ breeders and 2 tdTomato reporter LE rats. Thirty ChAT Cre+ rats are currently in process of operant training and tinnitus testing. As delineated by the block diagram at the top of Figure 1, rats were assigned into the three groups: C, T and NT animals. Following sound-exposure, brainstem evoked response threshold testing, all animals began 3 months of condition-suppression training followed by testing for the presence or absence of tinnitus. Thirty rats were designated as unexposed controls (C). Based on their condition suppression score 26 rats were designated as having tinnitus (T) and 25 were designated as sound-exposed non-tinnitus (NT). An additional 3 rats were eliminated from the study due to hearing problems. The largest number of rats (76) were designated for the *in vitro* electrophysiology/pharmacology studies in SA1B & SA1C described below. The remaining 6 rats were used in preliminary *in vivo* recording (SA2) and immunohistochemistry (SA1A) studies. Figure 2 shows results of tinnitus testing in a 16 kHz background for a subgroup of 20 rats (10C, 10T). The lower the suppression score across intensities the less the animals were suppressing to silence. The less they were

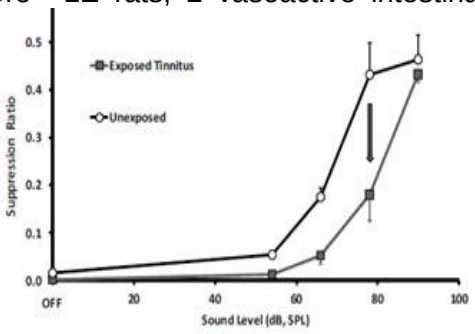

Figure 2. Classification of LE rats based on their suppression ratios across intensity as Controls or sound-exposed Tinnitus (16 kHz background). The lower the suppression ratio the greater the evidence of tinnitus. (n=20, 10C, 10T)

suppressing their lever pressing, the greater the likelihood they were hearing a phantom sound/tinnitus similar to the background frequency (Figure 2).

Accomplishment for year one by Specific Aim:

Year#1: Much progress was made during the first year which was focused on mechanistic aspects of attentional tinnitus pathology and preliminary drug studies. As will be detailed by specific aim below, we believe several enhancements (see Changes Section 5) have been made to the specific aims (SAs), which comprise the major goals of the project, using approaches not yet available at the time of its composition.

SA1A: Characterize tinnitus-related changes in nAChR subunit mRNA expression and composition of homomeric and heteromeric nAChRs in the major cell types across layers of AI in control and animals with behavioral evidence of tinnitus. *Hypothesis: Tinnitus will alter the composition and distribution of heteromeric α4β2 and homomeric a7 nAChRs across AI layers and cell types.*

Year#1: Studies described in SA1A have begun with the testing of antibodies in knock-out mice for the nAChR subunits to be examined. Extensive pilot immunoprecipitation studies have been carried out. Scheduling to develop, test, section and ship a group of 12 LE rats (4C, 4T and 4NT) to Professor Troy Hackett at Vanderbilt are underway as described in the original proposal and under changes/enhancements section 5 below. Professor Hackett will employ 8 or 12-plex FISH instead of the 4-plex FISH originally proposed. This will provide rich additional new data to this study.

Year#1: Seventy-six LE rats from the three (C, T, NT) experimental groups were assigned to SA1B and SA1C during year one.

SA1B: Characterize tinnitus-related, dose-dependent changes in ACh's ability to evoke nAChR-mediated synaptic currents from AI LIV-VI neurons in whole-cell slice recordings from animals with and without behavioral evidence of tinnitus. *Hypothesis: Neurons from animals with behavioral evidence of tinnitus will show altered nAChR responses to different concentrations of acetylcholine (ACh).*

Year#1: Results from whole-cell voltage-clamped LV AI neurons from animals with and without behavioral evidence of tinnitus showed a number of significant tinnitus-related differences. *nAChR mediated cholinergic signaling was disrupted in animals with behavioral evidence of tinnitus.* There was a significant tinnitus-related increase in the number of spontanious excitatiory postsynaptic potentials (sEPC's) ($t(64) = 2.83$, $p = 0.007$) and peak sEPC amplitude ($t(61) = 2.43$, $p = 0.018$, t-test) in animals with chronic tinnitus (Figure 3). There was also tinnitus-related increase in number of action potentials evoked by injecting depolarizing (2X rheobase [RC]) current in the presence of bath application of ACh in animals with chronic tinnitus ($t(12) = 2.5$, $p = 0.028$, paired t-test) (Figure 4A). Increasing doses of puffed ACh produced an increasing postsynaptic response in control animals ($p = 0.006$, Pearson corelation) (Figure 4B). This response peaked at the lowest-dose of ACh (0.01 mM) in tinnitus animals showing a saturating nAChR response at higher doses of ACh in tinnitus animals (Figure 4B). This increased response to puffed low-dose 0.01 mM ACh in tinnitus animals may reflect a residual current mediated by low affinity highly desensitizing α7 nAChRs. To test this hypothesis, LV AI neurons were puffed with 0.1 mM ACh in the presence of α7 blocker methyllycaconitine citrate (MLA) and showed a significantly higher α7 current in animals with tinnitus (Figure 4C). In AI, α7, α4 and β2 were found to be the principal nAChR subunits, therefore, on-going studies will selectively block β2 containing nAChRs using DHβE, to validate the observed tinnitus-related upregulation of postsynaptic α7 nAChRs.

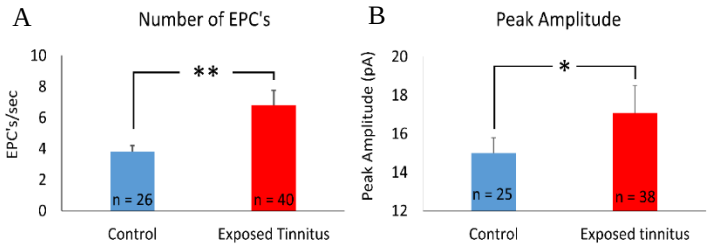

Figure 3. A significant increase in number of sEPC's ($t(64) = 2.83$, $p = 0.007$) and peak amplitude ($t(61) = 2.43$, $p = 0.018$, t-test) was observed in animals with chronic tinnitus.

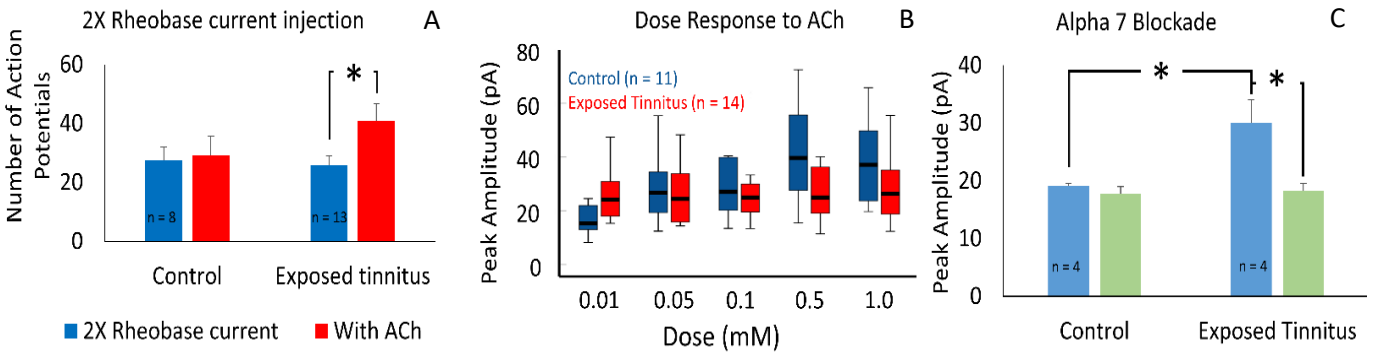

Figure 4. A) nAChR mediated cholinergic physiology was disrupted in LV pyramid cells from animals with chronic tinnitus. A significant increase in number of action potential was observed in response to 2X rheobase current in animals with chronic tinnitus ($t(12) = 2.5$, $p = 0.028$, paired t-test). B) Increasing doses of puffed ACh (0.01, 0.05, 0.1, 0.5, 1 mM) demonstrated relatively linear dose response in control animals. LV neurons from tinnitus animals showed a low dose response, which appeared to become desensitized at increasing concentrations of ACh (C, $p = 0.006$, T, ns). C) Response to 0.01 mM ACh from LV neurons in tinnitus rats was proportionally more sensitive to a7 blockade ($t(5) = 4.84$, $p = 0.005$, paired t-test)

*Acetylcholine exhibited altered **presynaptic** signaling in LV pyramidal cells:* Collectively control animals showed no differences in presynaptic sEPC's between baseline and optogenetic stimulation of basal forebrain (BF) cholinergic terminals (Figure 5A) and bath application of ACh (not shown). However, the heightened tinnitus-related sEPC baseline response was attenuated by optogenetic stimulation of BF cholinergic terminals (Figure 5A). Closer examination of the data revealed two patterns in the results which will be further examined in year two studies. Acetylcholine in a physiological setting may suppress non-essential spontaneous signals, while potentiating responses to meaningful signals.

Tinnitus upregulates thalamocortical signaling of LV pyramidal cells: Optogenetic stimulation of thalamocortical terminals evoked a significantly larger postsynaptic EPCs in animal's with chronic tinnitus, suggesting increased thalamocortical signaling. Post-synaptic EPCs consisted of a rapid brief excitatory signal followed by long and delayed inhibitory signal likely mediated by AI interneurons (not shown). Excitatory and inhibitory responses were significantly potentiated by bath application of 10.0 µM ACh (Figure 5B) in both control and tinnitus animals. This potentiation of thalamocortical input signals with bath ACh was non-significant, and will require additional examination in year 2.

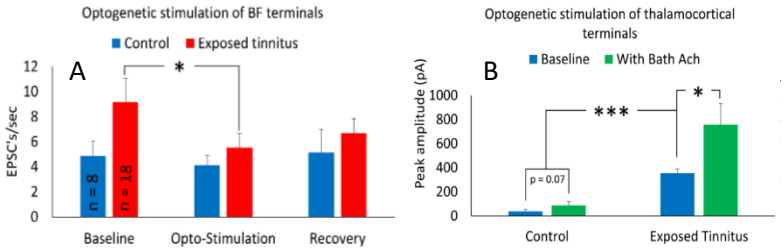

Figure 5. A) Acetylcholine exhibited bimodal response in LV pyramid cells, which was disrupted in animals with chronic tinnitus. Optogenetic release of ACh significantly decreased the number of sEPC's in animals with chronic tinnitus ($t(17) = 2.82$, $p = 0.012$). B) Optogenetic stimulation of thalamocortical terminals resulted in a significantly larger postsynaptic response relative to baseline ($t(9) = 7.82$, $p = 00002$, bath ACh, $t(9) = 3.42$, $p = 0.008$, t-test). B) n = 5C & 6T.

SA1C: Characterize synaptic currents of AI LIV-LVI neurons using pharmacological agents to probe tinnitus-related changes in the subunit composition of homomeric and heteromeric nAChRs in whole-cell slice recordings from animals with and without behavioral evidence of tinnitus. *Hypothesis: Saturation with optimized dose of varenicline tartrate (VCL) or sazetidine A dihydrochloride (Saz-A) reverses/ameliorates the dysregulated response properties of AI LV pyramidal neurons to acetylcholine and thalamocortical input signals.* Whole-cell voltage-clamped AI LV neurons were tested with the partially selective therapeutic agonists, Saz-A and VCL, as well as selective and non-selective nAChR antagonists (DHβE and mecamylamine) from control and animals with behavioral evidence of tinnitus. *Saz-A and VCL show differential actions.*

Year#1: When 1 µM Saz-A was bath applied for 10 minutes following acquisition of a continuous recordings baseline, Saz-A (1 µM) initially increased the number of sEPC's followed by a decrease at later time intervals. Saz-A was found to suppress and stabilize sEPC's in animals with chronic tinnitus. Close examination of resting membrane potential (RMP) showed significant hyperpolarization ($t(10) = 5.26$, $p = 0.0003$, paired *t*-test) and an increase in RC threshold/action potential threshold of AI LV cells during bath application of Saz-A (Figure 6A&6B). Compared to ACh, Saz-A evoked only small or no postsynaptic responses from LV AI cells (Figure 6C). When ACh was puffed onto a cell following Saz-A application the initial ACh response was significantly attenuated, presumably by Saz-A desensitization (Figure 6C). In the presence of atropine, patch clamp preliminary recordings from AI LV pyramid cells showed that 1 µM Saz-A may decrease the number of sEPC's in animals with behavioral evidence of tinnitus (Figure 6D). The number of sEPC's remained relatively stable throughout the test period

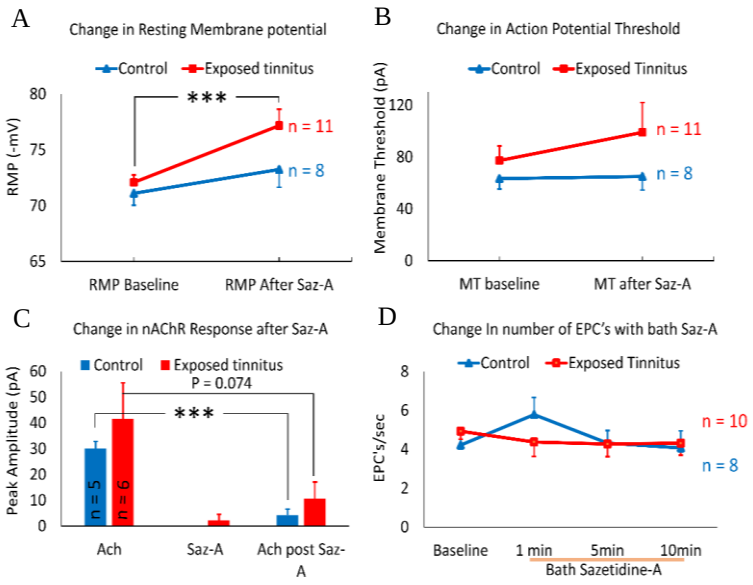

Figure 6. A) Saz-A decreased the sEPC's number stabilizing LV pyramid cell in a desensitized state: Saz-A significantly decreased RMP ($t(10) = 5.26$, $p = 0.0003$, paired *t*-test). B) RC was increased (non-significant) in animals with chronic tinnitus. C) Puffed Saz-A produced little to no response, followed by desensitization of nAChRs to puff ACh in control ($p = 0.0004$) and tinnitus animals ($p = 0.074$). D) Bath application of Saz-A initially increased, followed by decrease in number of sEPC's in control animals, while tinnitus animals showed a decrease in number of sEPC's.

This stabilized response may result from desensitization of α4β2 nAChRs, preventing generation of sEPC's. These results suggest Saz-A's action on nAChRs potentially may normalize tinnitus-related changes in AI LV pyramid neurons by stabilizing LV neurons in a partially desensitized hyperpolarized state, inhibiting the generation of sEPC's.

Proposed studies will:

SA2: Determine the tinnitus-related changes that occur in the discharge properties of single-units from AI LIV-LVI and the ability of nAChR agents to modify these responses. *Hypothesis: 1) Spontaneous, bursting, and driven activity will be significantly enhanced in animals with behavioral evidence of tinnitus. 2) Systemically administered selective nAChR partial agonists sazetidine-A and varenicline will normalize tinnitus-related response abnormalities from single-units from AI LIV-LVI.* Studies in **awake rats** will use advanceble tetrodes implanted in AI to record single-units (Kalappa *et al.*, 2014; Cai *et al.*, 2016). Following collection of data from C and T animals, experiments will include sazetidine-A or varenicline systemically administered one hour prior to recordings sessions.

Year#1: During year 1 we successfully piloted recording from AI units in two awake rats using our Neuralynx Versadrive 16 and 32 channel electrodes similar to our previous recordings from auditory thalamus (Kalappa *et al.*, 2014; Cai *et al.*, 2016). A number of animals in the present tinnitus production pipeline will be assigned to SA2. We would expect all of the physiologic and some of the pharmacologic studies outlined in SA2 will be completed by the end of year 2.

SA3A: Determine the ability of the nAChR partial agonist, varenicline, to ameliorate tinnitus in a sound-exposure operant rat model of tinnitus. *Hypothesis: Consistent with our preliminary findings with sazetidine-A (Figure 2, original grant proposal), evidence of tinnitus will be reduced by varenicline in animals with behavioral evidence of tinnitus.* Animals with behavioral evidence of tinnitus will be administered subcutaneous injections of different doses of varenicline one hour prior to tinnitus testing. Each dose will be given for one week followed by a recovery/washout period to allow evidence of tinnitus to reappear prior to testing additional doses.

Year#1: SA3A was not started during year one. SA3A will begin by the end of year 2.

SA3B: Determine the ability of the nAChR partial agonists, sazetidine-A and varenicline, to normalize tinnitus-related selective attention deficits seen in a new auditory selective attention task. *Hypothesis: Sazetidine-A and varenicline will improve selective attention performance in animals with behavioral evidence of tinnitus.* A selective attention task detailed below found tinnitus-related deficits in animals with behavioral evidence of tinnitus (Brozoski *et al.*, 2019); Figure 6, original grant proposal). Sazetidine-A or varenicline will be administered subcutaneously one hour prior to selective attention testing sessions.

Year#1: SA3B was not started during year one. These studies will begin early in year 3.

4. Impact

The results form SA1, delineated above, are the first of their kind to show significant tinnitus-related attentional abnormalities in principal AI output neurons. First they show significant abnormalities in the number and size of the presynaptic excitatory messages arriving at these neurons. Secondly they show significant tinnitus-related differences in nAChR sensitivity at different doses of acetylcholine. Since we will be pharmacologically targeting these receptors in attempts to normalize their tinnitus pathology, understanding these tinnitus-related differences is critical. The ability of sazetidine-A to effect disproportional changes in resting membrane potential and action potential threshold in animals with behavioral evidence of tinnitus suggests a unique receptor target. Finally, preliminary data suggests that sazetidine-A was able to normalize a tinnitus-related increased sensitivity to acetylcholine. Collectively, we feel these are exciting and highly publishable findings when matured. They also support the proposed SA2&3 studies.

5. Changes

Much progress was made during the first year in which we were focused on mechanistic aspects of attentional tinnitus pathology. We believe several enhancements have been made to the specific aims (SAs) of PR 180160 using approaches not yet available to us at the time of its composition.

Beginning just prior to year 1, we chose to modernize the Bauer/Brozoski (DOS-based with old/DIY interfaces) behavioral tinnitus training/testing system used to generate experimental subjects for this award. This system upgrade, using Lafayette Instruments hardware and software, increased our ability to generate tinnitus animals and saved us significant the consultant monies originally slated for Dr. Brazoski which are now only needed for emergency consulting as opposed to maintenance. We intend to use these consultant monies to enhance the neurochemical and electrophysiologic studies in SA1. These are not new activities within SA1, just enhanced. Preliminary *in situ* (FISH) data completed during the first year of this award used animals processed prior to start of this award. These initial studies found significant tinnitus-related differences in the distribution of 3 nAChR subunits in primary auditory cortex (AI), supporting the value of this approach. Based on these preliminary data obtained for SA1 and findings contained in our recent publication on aging and nAChRs (Ghimire *et al.*, 2020), will expand our collaboration with Professor Troy Hackett of Vanderbilt University to include more cholinergic and cellular markers/probes while also adding two additional auditory structures; auditory thalamus and the inferior colliculus to the proposed AI studies. These studies will utilize tissue from 4C, 4T and 4NT rats to be sacrificed and sectioned at SIU-SM with frozen sections transported to Dr. Hackett at Vanderbilt. This portion of SA1A will be completed during the second year of the study.

The electrophysiology studies benefit from the availability of genetic models. In addition to original proposed ChAT-Cre colony, we have added VIP-Cre Long-Evans Rats to our study. ChAT-Cre and VIP-Cre models allow us to use selective viral probes for our proposed *in vitro* and *in vivo* studies, and better manipulate the cholinergic attentional system in our tinnitus electrophysiological studies. The saved contractual monies will be used purchase the breeding licenses for these two Cre-strains of LE rats.

6. Products None Yet

7. Participants & Other Collaborating Organizations

Professor Troy Hackett at Vanderbilt University is a consultant to this project on the original award. His role has expanded well within the original framework and costs of the proposal as explained section 5 above.

8. Special Reporting Requirements None

9. Appendices None

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