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Identifying Molecular Pathways Underlying Noise-Induced Tinnitus

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Introduction

Subjective tinnitus is a potentially serious neurological condition affecting 10-15% of the general population (Bhatt et al., 2016; Tunkel et al., 2014). It is considered a symptom that is commonly associated with hearing loss including noise-induced hearing loss (NIHL). Subjective tinnitus is the perception of intermittent or constant “ringing” buzzing” or “hissing” sounds in the absence of an external sound source. The incidence of tinnitus increases during aging and is particularly prevalent in veterans due to NIHL. Patients suffering from tinnitus often experience difficulty concentrating, sleep deprivation, anxiety, and depression. (Hall et al. 2011), making it a debilitating condition. Currently there are no FDA approved drugs to prevent or treat tinnitus, mainly due to a lack of understanding of its cellular and molecular pathways.

Evidence obtained from both animal and human studies suggest that tinnitus results from the dysfunction of several neuronal networks encompassing both auditory and non-auditory pathways. Acoustic trauma is capable of damaging cochlear hair cells and auditory nerve fibers that can lead to a reduction of auditory inputs. In response to this cochlear deafferentation, the central auditory system appears to increase neural gain to over-compensate for the reduced sensorineural input from the cochlea. Animal studies suggests that NIHL causes hyperactivity in multiple areas of traditional and non-traditional auditory pathways (e.g., Bauer, 2004; Eggermont and Roberts, 2012; Kaltenbach, 2011). Animals with behavioral evidence of tinnitus typically exhibit increased spontaneous firing rates, abnormally high synchrony, and burst firing in the dorsal and ventral subdivisions of the cochlear nucleus, as well as the inferior colliculus (IC). This could subsequently lead to a reorganization of tonotopic maps within the auditory cortex structures (Noble and Tyler, 2009; Vogler et al., 2011; Robertson and

Mulders, 2012; De Ridder et al., 2014; Schoisswohl et al., 2019). These findings suggest that phantom percepts of sound arise from cochlear deafferentation, and reach awareness only when the hyperactivity in the auditory cortex is linked to a larger network that supports perception.

In the central nervous system, an imbalance between excitatory and inhibitory neural activities can lead to pathological conditions such as tinnitus, schizophrenia, autism, and Alzheimer disease. Dysfunctions of inhibitory neurons, mainly neurons that produce and respond to gamma-aminobutyric acid (GABA), are implicated in tinnitus. In addition, tinnitus is associated with an increase in spontaneous activity and sound-evoked activity in the IC following NIHL (Richardson et al. 2012; Longenecker and Galazyuk, 2011; Ma et al., 2006; Mulders and Robertson, 2011; Mulders et al., 2011). Along with increased spontaneous activity, a decrease in the firing of GABA neurons is also found (Richardson et al., 2012; Dong et al. 2010). Glutamic acid decarboxylase (GAD) is an enzyme in the brain that catalyzes the synthesis of GABA. Decreased mRNA expression of GAD has been shown to be linked to other related neurological disorders such as schizophrenia, bipolar disorder, and autism (Akbarian and Huang 2006).

Besides GABA dysregulation, several molecular pathways could be involved in tinnitus. The T-type calcium channels generate synchronized oscillatory activity in thalamocortical circuits through calcium-dependent low-threshold spikes, which underlie thalamic burst firing (Cheong and Shin, 2013). Among three T-type calcium channels (CaV3.1, CaV3.2, and CaV3.3), CaV3.1 is richly expressed in thalamic neurons, and plays a central role in the generation of thalamocortical rhythms (Cain and Snutch,

2013; Kim et al., 2001; Choi et al., 2015). Abnormal thalamocortical activity can be attributed to the disruption of coherent oscillatory activity between thalamus and cortex following hearing loss (Eggermont and Roberts, 2012; Eggermont and Tass, 2015). In addition, the hyperexcitability of auditory circuits may potentially be due to other molecular targets such as L-type calcium channels. Excess calcium influx into neurons in auditory pathways may be attributed to the underlying mechanisms of tinnitus (Monzani et al., 2015). Based on these previous studies, we tested a drug candidate, tetrandrine (TET), which can block both T-type and L-type calcium channels, and found that it was effective in treating noise-induced tinnitus in our animal model (Zuo et al., 2017). TET is a drug approved in China for silicosis, hypertension, inflammation, and lung cancer with a long history of good safety profile (Xie et al., 2002). Here, based on this interesting finding. TET was further tested, along with other calcium channel antagonists, to reveal possible molecular mechanisms of noise-induced tinnitus, and explore new molecular targets to treat tinnitus.

Both animal and clinical studies have shown that certain drug candidates were promising in treating tinnitus, however the molecular target and mechanisms remain unclear, with most of them are involved in calcium signaling. Gabapentin (GAB) has been initially found to be 50% effective in reducing psychoacoustic loudness in patients (Richardson et al., 2012; Bauer and Brozoski 2006). GAB was designed to mimic the neurotransmitter GABA; however it does not bind to GABA receptors. Its mechanism of action as an antiepileptic agent likely involves its inhibition of the alpha 2-delta subunit of voltage-gated calcium channels, which is critical for the function of L-type calcium channels. Similarly, ethosuximide (ESM) is commonly prescribed to treat seizures and

acts as a T-type calcium channel blocker (Browne et al., 1975). Nimodipine (NMDP) is an FDA approved L-type calcium channel blocker developed to treat high blood pressure and has also been found to be effective in labyrinthine dysfunction (Monzani, 2015; Lassen et al., 1996; Pianese et al., 2002), dizziness (Monzani, 2015; Wu et al., 2013), and tinnitus (Monzani et al., 2015; Davies et al., 1994; Monzani et al., 2012). While not many recent studies have further investigated the use of NMDP in treating tinnitus, past clinical studies have demonstrated that it might be effective (Davies et al., 1994). Here, I examined possible effects of these drug candidates in order to determine possible molecular signaling for tinnitus. This preliminary data will inform our next steps in the identification of effective molecular targets for tinnitus treatment.

Materials and Methods

The procedures were completed in accordance with the Northeast Ohio Medical University Institutional Animal Care and Use Committee. A total of 13 C57/BL/6J mice (9 male/4 females) were included in the study and underwent behavioral training for tinnitus detection, tinnitus induction, and treatment application and efficacy measures. The sound-based avoidance detection (SBAD) method was used for detecting tinnitus and testing the response to different pharmacological doses (Zuo et al., 2017). Traumatic noise exposure was used to induce tinnitus in the mice.

Behavioral Training and Testing for Tinnitus Detection: Experimental mice were placed in a shuttle box that was divided into two compartments. Mice were trained to cross from side to side when sound cues were presented through MF1 speakers (“Go” trial). Mice were trained to remain still when no sound was presented in the shuttle box (“No-Go” trial). This training was performed over 15 days. The animals were given a 15-30

minute acclimation period in the shuttle box before training began. A training session included 100 randomly assigned trials per day, lasting approximately 30-40 minutes per session. The Go trial sound cues were randomly played as white noise or narrow-band noise at center frequencies of 8, 16, 20, 32, and 40 kHz, and pure tones of 8, 16, 20, 32, and 40 kHz, all of which were presented at random intensities ranging from 85-100 decibels sound pressure level (dB SPL) at 5 dB increments. To reinforce the training sessions, the mice were shocked if they did not cross from one compartment to the other during the Go trials. They were also shocked if they crossed sides during the No-Go trials. After the 15th training day, mice were tested for 3 days to obtain three baseline scores. Testing procedures were repeated after noise exposure to be compared with baseline scores. All baseline and post noise exposure testing sessions presented randomized 90 Go trials and 30 No-Go trials with shocks enabled during the Go trials but disabled during No-Go trials.

Tinnitus Induction: Following the successful completion of training (>/= 90% correct rate for Go and No-Go trials), and baseline testing for 8 mice (4 males and 4 females) at approximately 5 months of age and 5 mice (All males) at approximately 3 months of age, the mice were re-trained for three consecutive days. They were then exposed to traumatic noise levels over a two-day period to induce tinnitus. To protect the mice from bilateral NIHL which would affect the performance during Go trials, one ear was occluded with an ear plug, while the other ear was exposed to the noise. The mice were placed in a sound booth where they were exposed to a constant 116 dB SPL broadband noise (4-25 kHz) for two hours. Following the noise exposure, the mice were housed for one month, and then tested to see if they have developed tinnitus. To determine if an

animal exhibited successful tinnitus induction, Chi-Squared tests using a α level of 0.05 were computed to compare no-go scores at baseline and post-exposure. A statistically significant reduction in No-Go scores post-noise exposure compared to baseline was considered an indication for tinnitus. If tinnitus was not present in the mice one-month post-noise exposure, testing procedures were repeated after one month. This continued to up to 3 months (4 total months post noise exposure).

Pharmacological testing: The drugs that were tested included TET, GAB, ESM, and NMDP. Saline was also included to characterize the stress-related effects of animal handling, restraint and needle injections that could affect Go and No-Go performance. Mice that tested positive for tinnitus (n=6), underwent a series of treatment regimens that involved various drug combinations and dosages. Appropriate drug dosages used in this study were determined by previous preliminary work completed at this lab. Mice were weighed the day of testing to calculate drug volume. The mice were tested 2 hours after every intraperitoneal (IP) injection. GAB and ESM were dissolved by saline, while TET was dissolved in dimethyl formamide. NMDP was dissolved using corn oil.

Similar to our tinnitus detection statistical analysis, efficacy of all treatment regimens were determined by chi-squared testing with an α level of .05. If there was a statistically significant improvement in No-Go scores post treatment compared to post-noise exposure, it would indicate successful tinnitus treatment. All statistical analyses were computed using Jeffrey's Amazing Statistical Package (JASP) 0.9.0.1.

Results

Chi-squared tests were first used to determine if mice were tinnitus positive by comparing the baseline No-Go scores to post-noise exposure No-Go scores. A significant difference ($p < 0.05$) of the test indicated a tinnitus positive mouse. 6 mice (4 males and 2 females) were found to be tinnitus positive for a success rate of 46% out of the 13 mice trained. Following this analysis, the mice were tested accordingly to determine if their No-Go scores could be improved by different drug doses and combinations. Preliminary data indicated that TET dosages of 60 mg/kg and 30 mg/kg were effective in treating tinnitus positive mice, while a dose of 15 mg/kg did not significantly improve the No-Go scores (**Figure 1**).

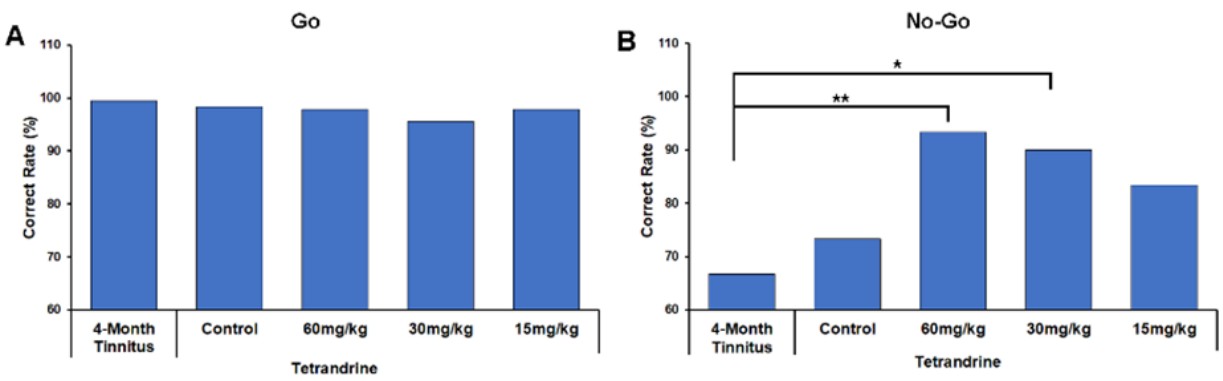

Figure 1. Preliminary data on the effects on different dosages of TET in tinnitus Go (A) and No-Go (B) scores. Dosages of 60 mg/kg, 30 mg/kg, and 15 mg/kg were used to determine the effectiveness of TET in treating tinnitus, with the 60 and 30 mg/kg doses leading to significant differences, and the 15 mg/kg dose having no significant difference.

Three months post noise exposure, two mice (11060-1 and 11060-2 in **Figure 2**) exhibited no statistically significant change in the No-Go scores from all three baselines ($p > .31$). While one mouse (11061-3) showed a significant drop of 26.67% in its No-Go score on day 3 of testing 3 months after noise exposure ($p = 0.002$), this was not

enough to be considered an indication of tinnitus. In this mouse (11061-3), no statistically significant changes were exhibited in the Go-scores from baseline in all 3-month post noise exposure scores ($p > 0.31$), while one mouse (11060-1) had a significant drop in its Go-score on day 3 of testing ($p < 0.001$). One mouse (11060-2) had statistically significant drops in its Go-scores on days 2 and 3 of testing (p </= 0.03), possibly due to hearing loss following noise exposure.

The 3 control mice were tested with GAB at 40 mg/kg, ESM at 300 mg/kg, and NMDP at 30 mg/kg to observe the possible behavioral effects of these drugs. On average, GAB did not have a significant change in the Go-scores of the mice with an increase of 2.1%, as well as NMDP with an increase of 1.36%. ESM did cause a significant difference in the mice with a decrease of 6.05% in the Go scores (**Figure 3a**). Individually, only one of the three animals exhibited a significant change in the go performance post ESM application (11061-1 in Figure 3a; 18.89% decrease, p=0.002). The average change in No-Go scores for all tested drugs ranged from 3.33% to 4.43%, all of which were not significant. No significant effects on No-Go performance were observed from each individual animal (**Figure 3b**). We therefore concluded that the tested dosages were appropriate for testing.

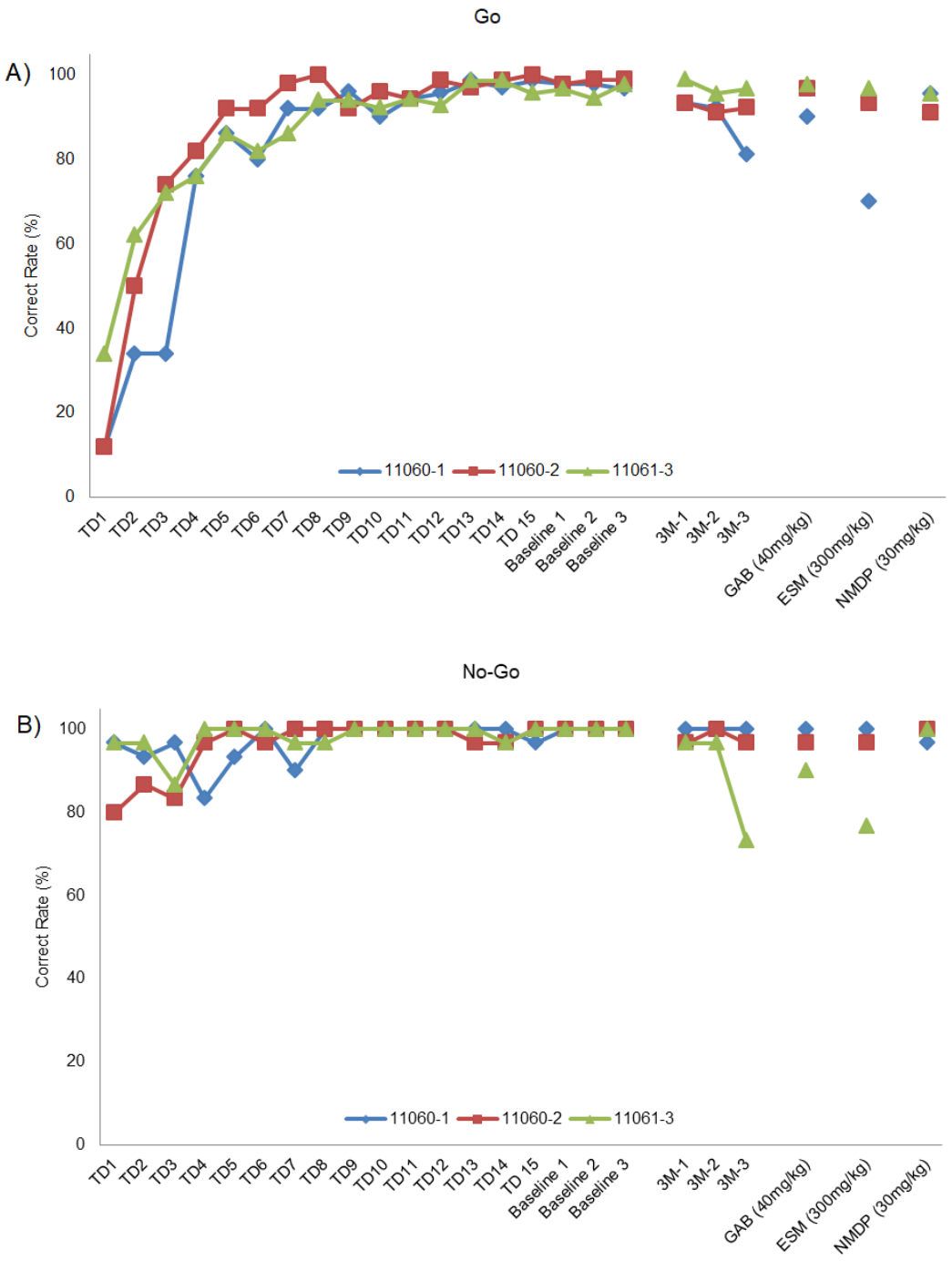

Figure 2. Go (A) and No-Go (B) training and testing correct rates (%) for control group. Mouse 11061-3 had a No-Go score significantly different from the baseline only on day 3 of testing post-noise trauma after 3 months, however the remaining days were all tinnitus negative. Drug testing was carried out to observe behavioral effects at different concentrations. TD = training day; 3M = three months post noise exposure.

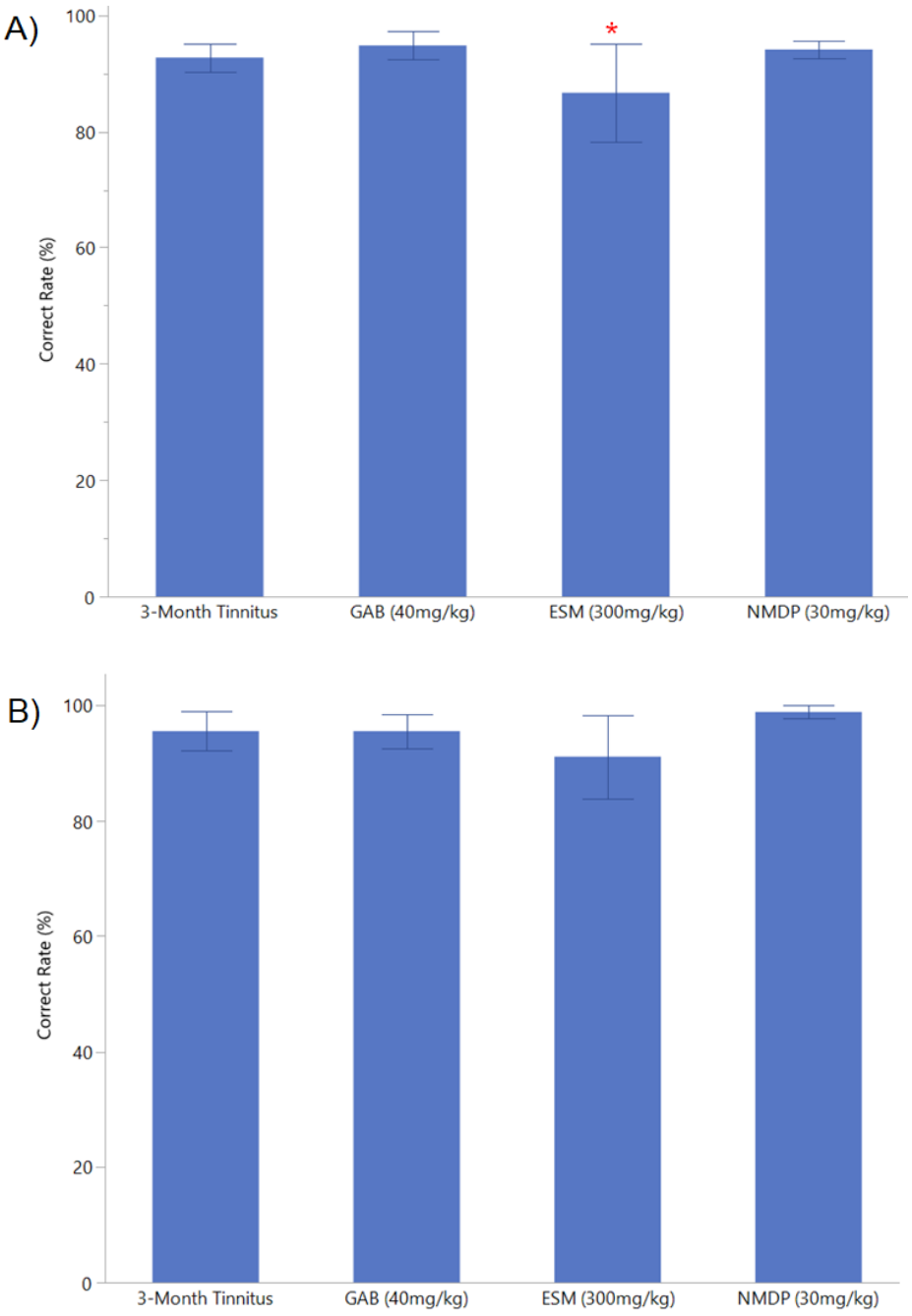

Figure 3. Averaged drug testing results of control mice (11060-1, 11060-2, and 11061-3) for correct rates (%) ofGo (A) and No-Go (B) test trials. 3 tinnitus tests were compared with the drug tests results. There was a significant difference in average performance of Go trials when tested with ESM (300mg/kg). While this average change in score was significant, only one animal in the group (11061-3) yielded a significant reduction in the Go score (*$p < .05$).

Group 1 included three animals that were successfully trained and tested positive for tinnitus post noise exposure (**Figure 4**). One mouse (11060-3), had significant decreases in No-Go scores on day 2 and 3 of 3-month tinnitus testing with decreases ranging approximately 23% to 27% (p </= 0.005,). One mouse had no significant differences on all 3 days of testing (p = 0.313, 0.161, 0.448). However, it did subsequently exhibit hyperactive behaviors consist with other tinnitus mice. One mouse had significant decreases in No-Go scores on day 1, 2, and 3 of testing, with decreases ranging from approximately 23% to 37% (p </= 0.005).

On average for Group 1, the Go-Scores (**Figure 5A**) significantly decreased in two of the following treatments: TET 60mg/kg (p = 0.02) and TET 60mg/kg+GAB 40mg/kg (p = 0.006) while they significantly increased in treatment with GAB at 40 mg/kg (p = 0.02). Individually, significant decreases were seen in one mouse (11061-1) treated with TET 60mg/kg (p = 0.03) and one mouse (11060-3) treated with TET 60mg/kg+GAB 40mg/kg (p = < .001). In the averaged No-Go scores (**Figure 5B**), 4 of the treatments had significant improvements, which included TET 60mg/kg (p < .001), TET 60mg/kg+ GAB40mg/kg (p < .001), NMDP 30mg/kg (p < .001), and TET 10mg/kg+NMDP 10mg/kg (p < .001). Individually, two of the animals exhibited significant improvements in No-Go scores for the treatment of TET 60mg/kg (11060-3, p = 0.04; 11061-1, p < .001), TET 60mg/kg+ GAB 40mg/kg (11060-3, p = 0.01; 11061-1, p < .001), and NMDP 30mg/kg (11060-3, p = 0.01; 11061-1, p < .001). Individually for the TET 10mg/kg+NMDP 10mg/kg treatment, one animal had significant improvement (11061-1, p < .001). In one of the treatments groups, ESM 300 mg/kg+GAB 40mg/kg, a significant decrease in No-Go scores was observed (p < .001). Additionally in the No-Go

scores, no significant difference was seen in the following treatments: GAB 40mg/kg (p = 0.382), ESM 300mg/kg (p = 0.298), ESM 300mg/kg+GAB 40mg/kg (p = 0.72), and NMDP 10mg/kg (p = 0.382). Refer to Figures 6 and 7 for Group 1 individual and averaged data respectively.

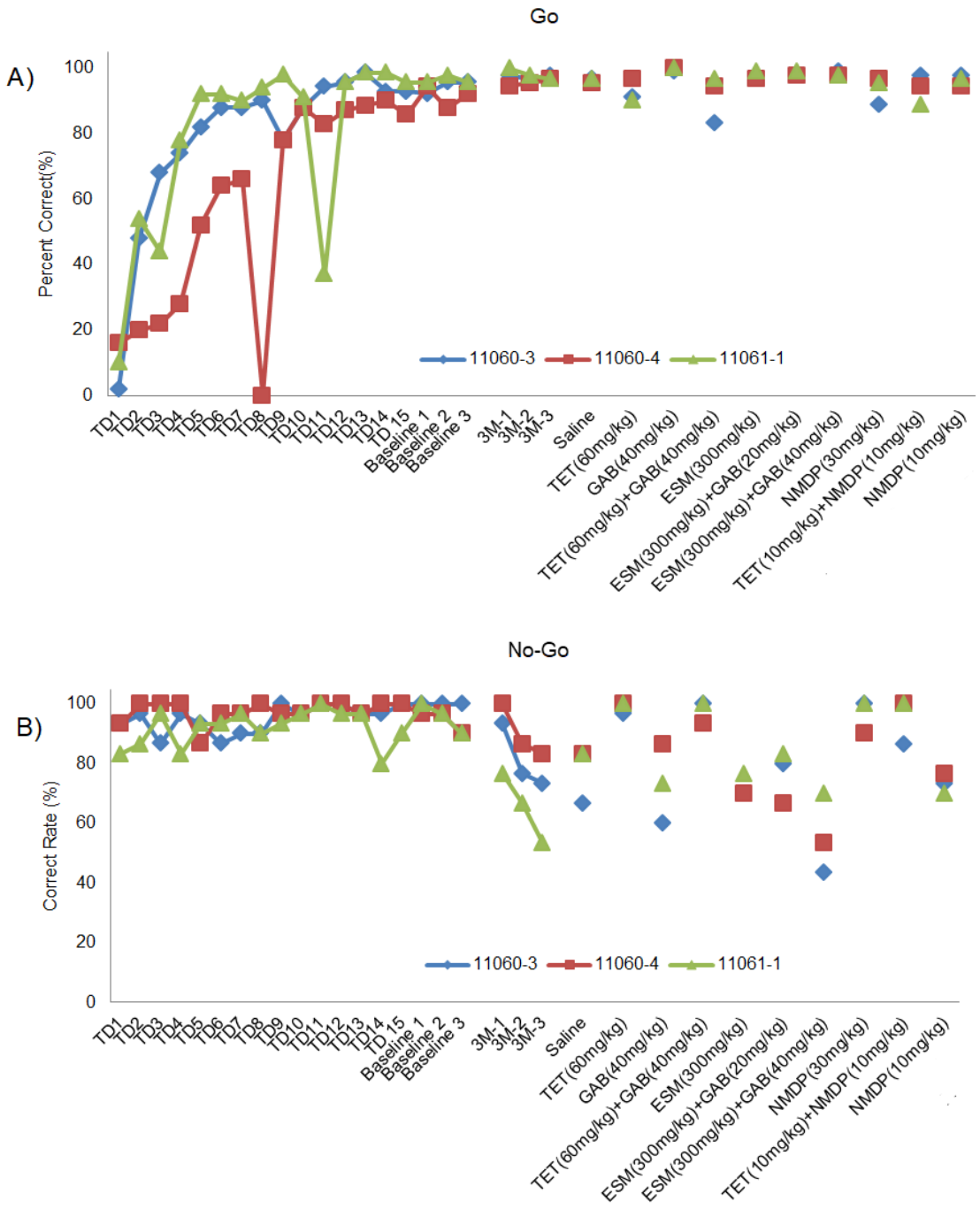

Figure 4. Go (A) and No-Go (B) training and testing correct rates (%) for Group 1. Two of the mice were found to be tinnitus positive after 3 months from noise exposure, while mouse 11060-4 was not, however it displayed erratic behavior during all three testing days and thus was included in Group 1. Drug testing was carried out with the specified drug concentrations and combinations. TD = training day, 3 M = three months post noise exposure.

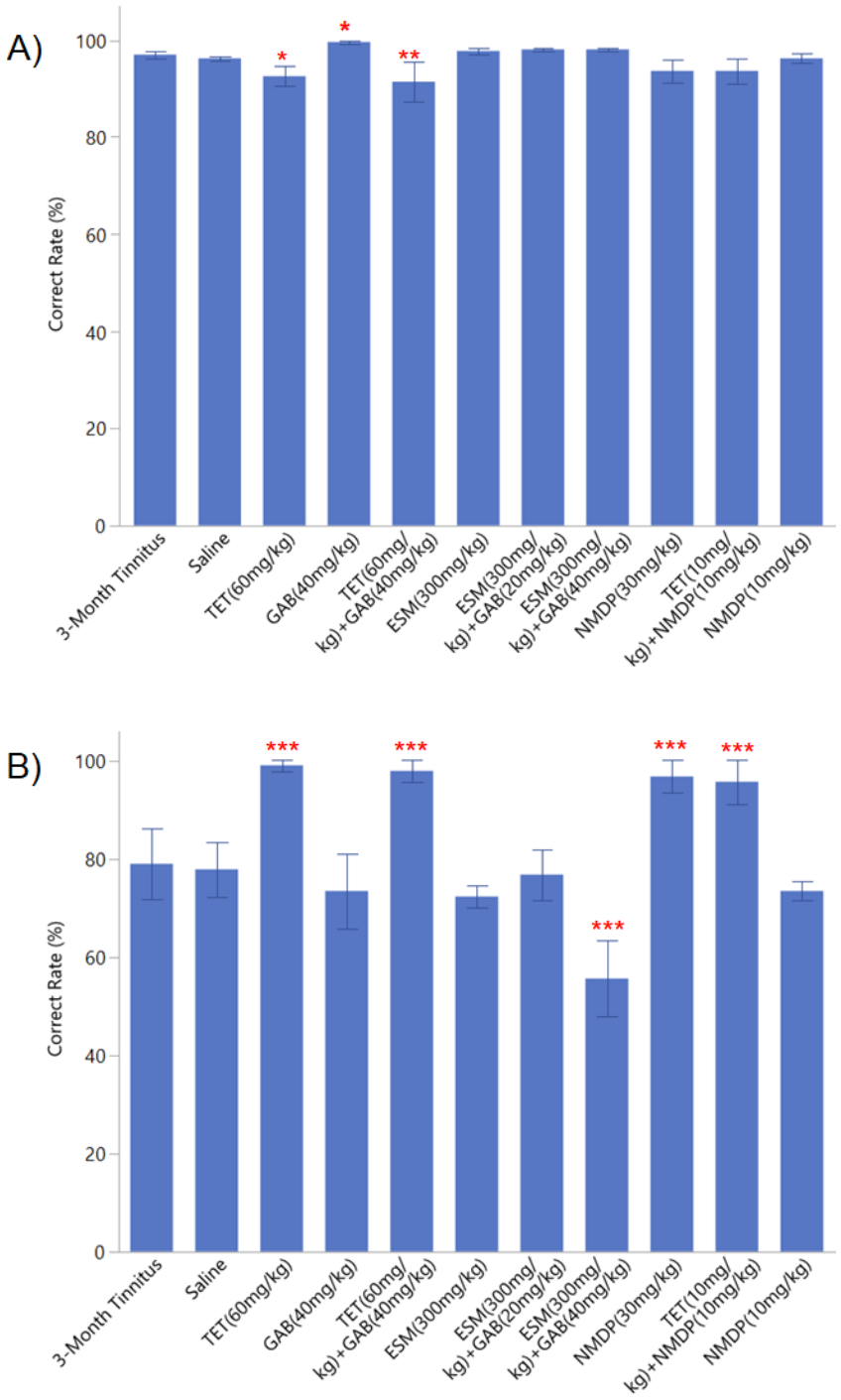

Figure 5. Average Go (A) and No-Go (B) correct rates (%) for Group 1. 3 month tinnitus tests were statistically compared to each individual treatment condition using chi-square tests (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Group 2 included three animals that were successfully trained and tested positive for tinnitus post noise exposure (**Figure 6**). Average Go scores for this group significantly decreased in three of six treatment conditions: saline (p = 0.002), GAB 80mg/kg (p = 0.03), and TET 60mg/kg +GAB 80mg/kg (p < 0.001). (**Figure 7A**). Average No-Go scores did not improve in any treatment condition for Group 2 (**Figure 7B**). Two treatment conditions (GAB 80mg/kg and GAB 40 mg/kg) yielded significant decreases in average No-Go scores (p < .001). While there was no significant improvement in average No-Go performance during the TET 60mg/kg+GAB 80mg/kg treatment condition, two of three animals exhibited significant improvements(11035-1; p = 0.02; 11036-1; p =0.002), while the third animals demonstrated significant decreases in its performance (11035-2; p = 0.003). In the saline treatment, average No-Go performance was not significantly affected; however one mouse (11035-2) exhibited significant decreases in its performance (p < 0.006).

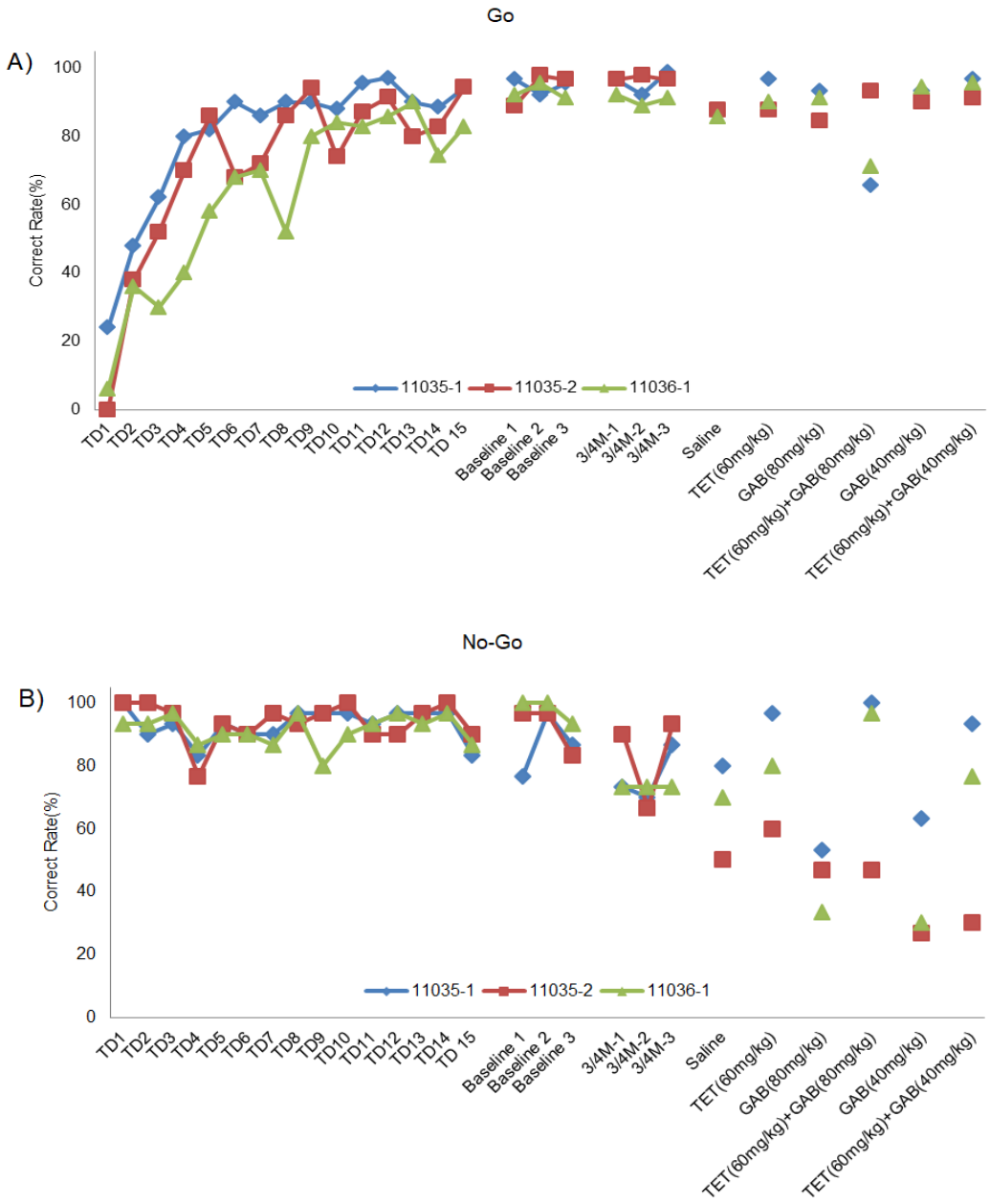

Figure 6. Go (A) and No-Go (B) training and testing correct rates (%) for Group 2. Mouse 11036-1 was tinnitus positive 3 months after noise exposure across all three testing days ($p </= 0.04$), while mice 11035-1 and 11035-2 were tinnitus positive across two and one testing day/s respectively after 4 months ($p </= 0.006$). While animal 11035-2 only demonstrated tinnitus-like behavior for only one out of three testing days post noise exposure, it displayed erratic behavior during all three testing days and thus was included in Group 2. Drug testing was carried out with the specified concentrations and combinations. TD = training day, 3/4 M = three- or four-months post noise exposure.

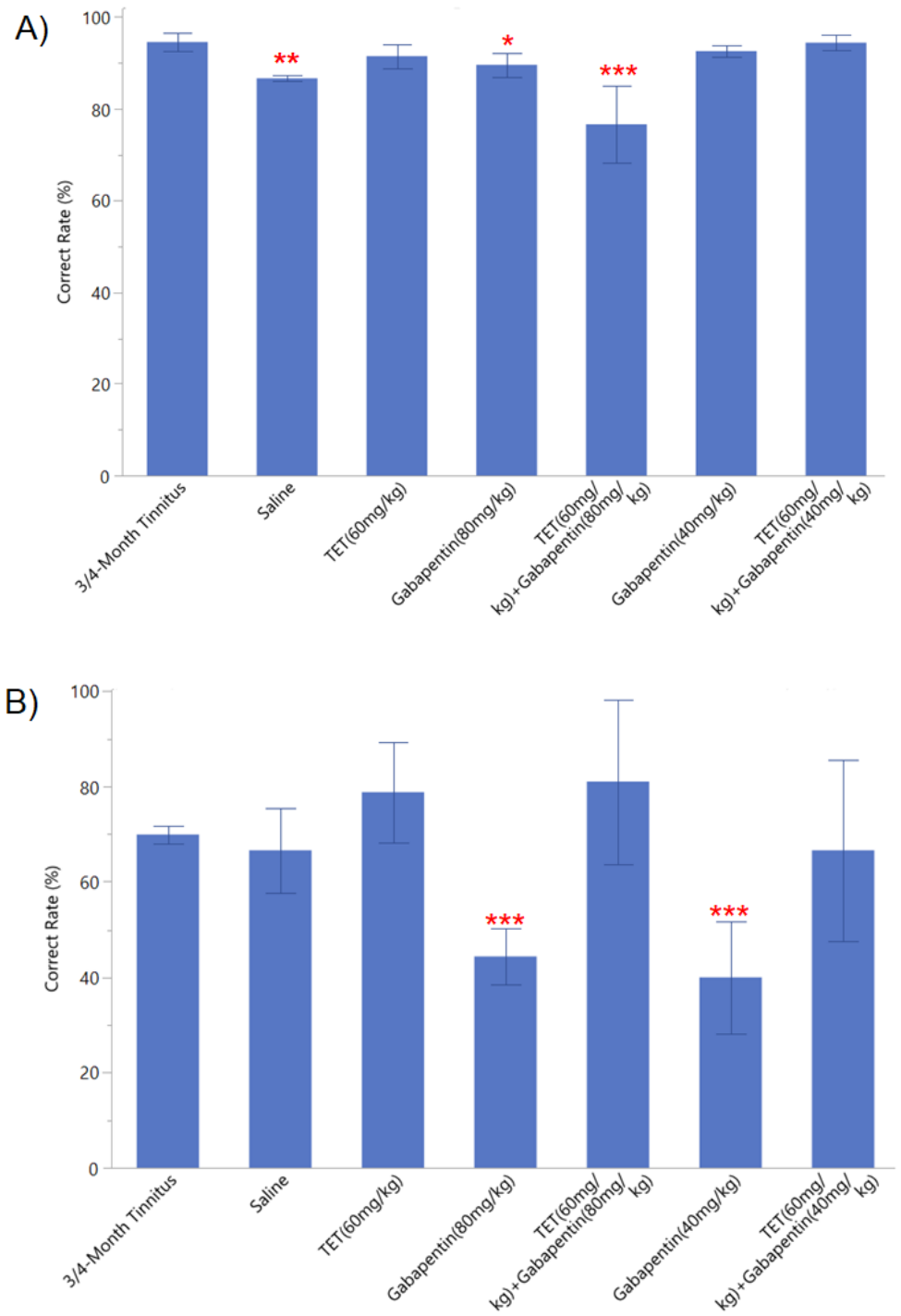

Figure 7. Average Go (A) and No-Go (B) correct rates (%) for Group 2. 3 and 4-month tinnitus tests were statistically compared to each individual treatment condition using chi-square tests (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

In summary of the drugs' effects on tinnitus animals, average Go scores in Group 1 were significantly decreased by treatments of TET 60mg/kg and TET 60mg/kg+GAB 40mg/kg, and significantly improved by GAB 40mg/kg (**Figure 5A**). The average No-Go scores in Group 1 significantly improved for the treatments of TET 60mg/kg, TET 60mg/kg+GAB 40mg/kg, NMDP 30 mg/kg, and TET 10mg/kg+NMDP 10mg/kg (**Figure 5B**). In Group 2, the average Go score significantly decreased with treatments of saline, GAB 80 mg/kg, and TET 60mg/kg+GAB 80 mg/kg (**Figure 7A**). The average No-Go scores for Group 2 significantly decreased with treatments of GAB 80mg/kg and GAB 40 mg/kg (**Figure 7B**).

Discussion

Dysregulation of calcium signaling in the central nervous system is associated with several neurological diseases. Based on our preliminary studies, we examined if calcium channels blockers could modify tinnitus in our noise-induced models. Our results strongly suggested that, in contrast to our original hypothesis, T-type calcium channels were most likely not involved in tinnitus pathology since its specific blocker, ESM, has no effects on tinnitus even at a high dosage. Interestingly, our data did implicate that L-type calcium channels might be involved: two blockers of L-type calcium channels (TET and NMDP) were effective to eliminate tinnitus. However, GAB, a blocker for high-voltage calcium channels including L-type calcium channels, could cause worsening tinnitus. Since high-voltage calcium channels also include other calcium channels such as P- and Q- channels. This unexpected result could be due to complicated GAB inhibitions of these different channels in the brain. Thus, based off

these initial findings, L-type channels may be involved in noise-induced tinnitus. It is also clear that TET alone can be an effective drug for treating tinnitus.

Both experimental groups displayed statistically significantly reduced average Go scores when injected with saline. It is possible that the injection process caused stress related factors that would affect SBAD performance, however it is worth noting that there were no significant changes in No-Go scores pre and post saline injection. Additionally, in Group 1, one mouse exhibited significant decreases in its Go scores when treated with TET (60 mg/kg), and another exhibited significant decreases when treated with TET (60 mg/kg)+GAB (40 mg/kg). Individually, the Go scores remained above 80%. Regarding the aged group (Group 2), when treated with TET (60 mg/kg) and combination therapy of TET (60 mg/kg) and GAB (80 mg/kg), there were significant reductions in Go scores. Individually however, the decreased Go scores post treatment were all above 80% for both therapies and there was no significant change in No-Go scores. This is in contrast with findings from Group 1 which displayed significantly improved averaged No-Go scores when treated with both treatment regimens. These discrepancies between age groups needs to be investigated further, but it is possible that age related decline of hearing, learning, and/or memory can affect behavioral data.

With our current pharmacological approach, we were not able to identify any brain structures involved in tinnitus. It is known that the IC activity is largely associated with the onset of tinnitus. It acts as a relay site coordinating descending and ascending auditory signaling from cortical and subcortical sources. As such it is believed to have an intricate system of inhibitory signaling necessary for modulation which is largely mediated by GABAergic neurons (e.g. Ito et al., 2009). Dysfunction of GABA neurons is

believed to be associated with increases in spontaneous activity that could lead to the perception of phantom sounds (Richardson et al. 2012; Longenecker and Galazyuk, 2011; Ma et al., 2006; Mulders and Robertson, 2011; Mulders et al., 201). In the IC, high voltage calcium channel currents are contributed to primarily by high threshold voltage-activated calcium channel types, including L-, N, and P-types, while low voltage threshold activated calcium channels such as T-type calcium channels have lower expression and do not seem to significantly contribute to the action potentials and neuronal firing of the IC (N'Gouemo and Morad, 2003). Thus, when using ESM to block the activity of the T-type calcium channels, the low contribution of T-type calcium channels in IC activity did not cause any significant changes, while blocking the L-type calcium channels had a significant effect due to its strong contribution to the overall calcium currents of the IC. In the future, I plan to use molecular techniques to directly quantify gene expressions of these channels in the ICs of mice with or without tinnitus, which would lead to a clearer picture on how these channels are involved in tinnitus.

There are several limitations in this study. First, our findings were obtained from a small number of mice positive for tinnitus due to the low-rate of noise-induced tinnitus. Due to a small sample size, we opted to include all tinnitus positive mice (including those that did not statically indicate tinnitus, but had behavioral signs of tinnitus) for analysis and they did consistently support the use of TET in treating tinnitus. However, a lower dose of TET should be explored in order to avoid possible behavioral changes. In the future, more strict criteria should be followed when testing animals to provide reliable and consistent results. The discrepancies of findings between our two experimental group highlights potential age-related effects such as hearing sensitivity,

memory and learning that could affect our behavioral data. Therefore, when using this experimental mouse model, we advise testing animals under the age of one year. Additionally, improving methods for noise-induced tinnitus or establishing new methods to induce tinnitus could increase the likelihood of mice developing tinnitus so that a larger sample size can be used. Other alternatives such as salicylate induced tinnitus may lead to more animals with temporal tinnitus, however it is not an accurate representation of most forms of tinnitus experienced by humans, as noise trauma and aging are believed to be more associated with the development and maintenance of subjective tinnitus in the general population.

Besides increasing our sample size to obtain stronger data, we will expand our studies to map new molecular targets for treating tinnitus and use different blockers and agonists of L-type calcium channels to confirm if L-type calcium channels are involved in tinnitus. Once a narrower list of molecular targets has been made, single molecular fluorescence in situ hybridization (smFISH) will be performed in order to analyze the gene expression of these certain targets in the brain.

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