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Research Paper

Glutamate is down-regulated and tinnitus loudness-levels decreased following *r*TMS over auditory cortex of the left hemisphere: A prospective randomized single-blinded sham-controlled cross-over study

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ABSTRACT

Using a prospective randomized single-blinded sham-controlled cross-over design, we studied the efficacy of low frequency (1-Hz) repetitive transcranial magnetic stimulation (*r*TMS) over auditory cortex of the left temporal lobe as an experimental treatment modality for noise-induced tinnitus. Pre/post outcome measures for sham *vs*. active *r*TMS conditions included differential changes in tinnitus loudness, self-perceived changes in the Tinnitus Handicap Questionnaire (THQ), and neurochemical changes of brain metabolite concentrations using single voxel proton magnetic resonance spectroscopy (^{1}H-MRS) obtained from left and right auditory cortical areas. While no subject in our sample had complete abatement of their tinnitus percept, active but not sham *r*TMS significantly reduced the loudness level of the tinnitus perception on the order of 4.5 dB; improved subscales in several content areas on the THQ, and down regulated (reduced) glutamate concentrations specific to the auditory cortex of the left temporal lobe that was stimulated.

In addition, significant pair-wise correlations were observed among questionnaire variables, metabolite variables, questionnaire-metabolite variables, and metabolite-loudness variables. As part of this correlation analysis, we demonstrate for the first time that active *r*TMS produced a down regulation in the excitatory neurotransmitter glutamate that was highly correlated ($r = 0.77$, $p < 0.05$) with a reduction in tinnitus loudness levels measured psychoacoustically with a magnitude estimation procedure. Overall, this study provides unique information on neurochemical, psychoacoustic, and questionnaire-related profiles which emphasizes the emerging fields of perceptual and cognitive MRS and provides a perspective on a new frontier in auditory and tinnitus-related research.

1. Introduction

Repetitive transcranial magnetic stimulation (*r*TMS) is one of several experimental procedures thought to be of value in suppressing chronic tinnitus by modulating neural activity underlying the stimulating magnetic coil. Based on theoretical considerations and relevant animal studies, low frequency *r*TMS (≤1 Hz) is thought to modulate cortical excitability via *inhibitory* mechanisms (e.g., Chen et al., 1997) whereas higher frequency *r*TMS stimulation (≥5 Hz), is thought to modulate *excitatory* mechanisms and reactive plasticity (Pascual-Leone et al., 1994; Wang et al., 1996). As a consequence, *r*TMS-based neuromodulation can influence underlying brain physiology and cognitive functions (Walsh

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and Cowey, 2000). Other non-invasive and/or invasive neuromodulation techniques utilized for tinnitus suppression and/or attempts to treat an individual's reaction to the tinnitus percept include: transcranial direct current stimulation, transcutaneous electrical nerve stimulation, cortical neurofeedback, electrical stimulation of auditory cortex, dorsolateral prefrontal cortex stimulation, deep-brain stimulation, and vagus-nerve stimulation paired with tones (e.g., Cheung and Larson, 2010; Vanneste and De Ridder, 2012; De Ridder et al., 2014, 2017; Tyler et al., 2017). However, based on systematic reviews of the available scientific literature, the data is mixed concerning the utility low-frequency *r*TMS as a means for tinnitus suppression (e.g., Meng et al., 2011; Peng et al., 2013; Lefaucheur et al., 2014). Furthermore, while *r*TMS as a treatment modality for tinnitus appears safe in the short term, long term treatment effects are not known (e.g., Muller et al., 2012; Peng et al., 2013).

Herein, the main thrust behind this work was to determine: if changes in tinnitus loudness measured psychoacoustically, if self-perceived changes associated with responses on the Tinnitus Handicap Questionnaire (THQ), and if changes in neurochemical concentrations of metabolites localized to auditory cortical areas in the left and right hemispheres using proton magnetic resonance spectroscopy (^{1}H-MRS) could be modulated with *r*TMS within the context of this experimental design. The design features of this experiment were in response to 5 sequential days of either active or sham *r*TMS over the left auditory cortex (20 min per day; 1200 pulses per session) using a prospective randomized single-blinded sham-controlled cross-over design.

2. Methods

2.1. Participants

Participants in this study were 30 males, ranging in age from 24 to 80 years (mean age: 54.2 years, SD: 14.2 years). These individuals were recruited from advertisements placed in area newspapers, by word-of-mouth, and/or referred from area professionals (physicians and audiologists). No restrictions were placed on gender, ethnicity, or socioeconomic status but participants were required to have a history of noise exposure as the probable inducer for their tinnitus and be ≥18 years and <90 years of age. This study was approved by the Institutional Review Board (IRB) of Wayne State University. All participants signed informed consent prior to entering the study and were paid for their participation.

A total of five individuals did not complete the entire study and therefore, were not used in this data analysis. In three people, work-related issues (unemployment and re-employment during the study period) were the underlying cause for not completing the study. In two others, MRI data files were corrupted due to equipment (computer) malfunction and these participants would not return to the MRI Research Facility for re-scanning.

2.2. Tinnitus perceptions

Of the remaining individuals that participated (n = 25), tinnitus descriptions ranged from a constant high-pitched tone, ringing, buzzing, humming, or hissing sounds, while others reported an alarm clock like sound, or cricket-like perceptions. Because of these complex individual perceptions, no attempt was made to match the frequency of the tinnitus by psychoacoustic methodology.

2.3. Inclusion criteria

All participants were required to have chronic tinnitus, defined operationally as being persistently present over the preceding 6-month period and scoring in the moderate or higher range on the Tinnitus Handicap Inventory (THI; score ≥38). Additionally, all participants were required to have a history of occupational, recreational, and/or military noise exposure; typically, but not always documented by pure-tone audiometry (i.e., a notch in the audiogram at or near 4 kHz) in one or both ears.

2.4. Exclusion criteria

Documented history of retro-cochlear or neurologic disease (acoustic tumors, brain tumors, strokes, demyelinating disease, etc.), history of epilepsy or seizures, metal in the head, neck, or chest regions, implanted devices that might be damaged in a high field magnetic environment (i.e., implanted ferrous electrodes, implants of various sorts, including: a cochlear implant, vagus-nerve stimulator module, cardiac pace maker, stents, certain heart valves, etc.), use of GABAergic agonist medications, and/or other types of pharmaceuticals used to treat depressive illness. Individuals with a history of “blast” exposures were also excluded.

We also note that other medications were being taken by individuals that were not deemed exclusionary by the criteria described above. We report this information to be as transparent as possible with respect to considering any pharmaceuticals that could potentially have impact on this experiment.

Overall, 13/25 (52%) of the participants were not taking any additional medications; however, 12/25 (48%) were taking one or more of the following pharmaceuticals, which included: 1 baby aspirin (81 mg) per day to reduce/prevent the possibility of myocardial infarction and/or stroke; and use of: Atorvastatin to treat high cholesterol levels; Ramapril or Linsinopril to treat high blood pressure; Metformin and Glipizide to treat Type II diabetes; Finesteride to treat benign prostrate hyperplasia; folic acid to treat a vitamin B3 deficiency; and multivitamins for general health-related maintenance.

2.5. Research design

A prospective randomized single-blinded sham-controlled cross-over design was used (see Fig. 1) for a graphic representation

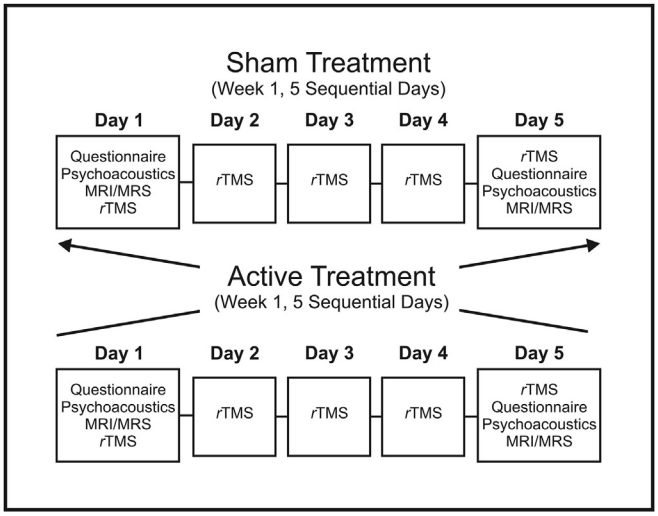

Fig. 1. Block diagram of the single-blinded sham-controlled cross-over design. Participants were randomly assigned to either the sham or active arm of the study in the initial phase. Then individuals underwent 5 sequential days of *r*TMS. During the first and fifth day, participants also completed the THQ, made psychoacoustical loudness judgments of their tinnitus, and had MRI/MRS testing performed.

of the study design. By single blinded, we mean that the experimenter but not the individual being studied was aware of the different experimental treatments. In one arm of the study, individuals were treated with active *r*TMS stimulation; in the other arm, individuals were treated with sham stimulation. Keeping sham and active stimulation protocols matched as closely as possible is an important component of these types of designs, such that the individual is not overtly informed, at least with respect to the device or stimulation protocol, to which condition they were assigned. Initial assignment to the active or sham condition was randomized. As we show in Appendix 1, input-output functions for the sham and active figure-of-eight coil acoustic click levels were ascertained such that the sound pressure level (SPL) could be equated (matched) between conditions. This was necessary because it was determined by subjective perceptual accounts, that the sham coil had much higher acoustic output levels than the active coil under identical power amplifier settings. In addition to equating for SPL differences, all other comparative features of the two figure-of-eight coils were identical, including: physical appearance (size, shape, color) and placement over the lateral surface of the temporal lobe. The only exception was the fact that one figure-of-eight coil was active and other was not with respect to magnetic stimulation.

The level used for active *r*TMS stimulation followed a protocol used in other studies, as described in the *r*TMS methods section. At the initial appointment and prior to any *r*TMS, individuals filled out the THQ, estimated the loudness of their tinnitus based on a psychoacoustic magnitude estimation procedure, and had high resolution structural MRI and MRS measures performed. Then, individuals received the *r*TMS/treatment. In this segment of the experiment, individuals were assigned randomly to receive either 5-sequential days of *sham* or 5-sequential days of *active* low frequency *r*TMS (20 min per day, at 1-Hz stimulation rate, totaling 1200 pulses per session). After the initial treatment (day 1), *r*TMS measures were repeated for the next 3 sequential sessions (days 2 through 4). On day 5, following completion of the questionnaire and psychophysical loudness measures of their tinnitus, the session ended with obtaining MRI and MRS. Following a washout period, which lasted no less than 2 weeks in duration (average: 3.28 weeks, SD: ±0.74 weeks, range: 2–5 weeks), this identical process was repeated and a cross over to the other arm of the experiment was implemented (sham-to-active or active-to-sham).

2.6. Questionnaire data

Two questionnaires were used in this study, the Tinnitus Handicap Inventory (THI; Newman et al., 1996) and the Tinnitus Handicap Questionnaire (THQ; Kuk et al., 1990). The THI was used as a screening tool to help select individuals for entry into the study in terms of identifying the severity of their handicap; where the following ratings could be ascertained: no handicap indicated by a score (0–16 points); mild handicap (18–36 points); moderate handicap (38–56 points); and severe handicap (58–100 points) (see Newman and Sandridge, 2006). Utilization of this questionnaire as an inclusion metric was based on its response requirements and ease of use. Indeed, response requirements of this questionnaire were straightforward and participants only needed to respond to individual queries by checking “yes,” “no,” or “sometimes” to each question or statement on a paper answer sheet. Thus, in its primary form, the THI consists of 25 questions and represents a “trinary” scale which is later modified by attaching numerical values to the individuals' responses in order to obtain an index on the severity-of-handicap. Furthermore, favorable psychometric operating characteristics were other essential reasons for selecting this metric. These included: high internal consistency reliability (Cronbach's alpha = 0.93), test-retest reliability for the total score (r = 0.92) and subscales scores ranging from r = 0.84 to r = 0.94 (Newman et al., 1998; also see Newman and Sandridge, 2006). In addition to ease-of-administration, the THI could be administered in a relatively short period of time, thus, further enhancing its value as a screening tool.

In addition to the THI, the THQ was chosen as an outcome measure, also based on ease-of-administration and favorable operating characteristics but primarily because it had a much larger range of values by which participants could rate their responses (0–100). Instructions for responding to each question/statement on the THQ were based on a numerical scale, where: 0 represents “strongly disagree” and 100 represents “strongly agree.” Numbers in between these values include subjective response magnitudes corresponding to an individual's agreement/disagreement with each statement posed. Furthermore, we also point out that the THQ has good operating characteristics. As noted by independent researchers, the THQ demonstrates high internal consistency reliability for the Total score (Cronbach's alpha = 0.95), Factor 1 (0.95), Factor 2 (0.88), but not Factor 3 (0.47) (Newman and Sandridge, 2006). Given these results, and since most of the variance was accounted for by the Total score; including Factors 1 and 2, only these three variables were recommended for data analysis purposes (Newman et al., 1995). The third factor was not included because of lower reliability scores. The Factor 1 subscale included questions dealing with social, emotional, and behavioral (physical) issues (SEB), the Factor 2 subscale included questions dealing with issues related to tinnitus and hearing (T&H), and Factor 3, although not included, dealt with one's outlook on tinnitus. Test-retest stability over a 6-week period demonstrated high correlations for the Total score (r = 0.89), Factor 1 (r = 0.89) and Factor 2 (r = 0.90). Reliability for Factor 3 was deemed inadequate (r = 0.50) (Newman et al., 1995). These criteria are consistent with the view of experts that for use in clinical assessment, test-retest correlations above 0.80 are considered good whereas test-retest correlations below 0.70, are generally considered unacceptable (e.g., Charter, 2003; Cicchetti, 1994).

2.7. Audiological data

Standard audiometric testing was conducted in a commercial sound booth (Acoustic Systems, Austin, Texas; Model RE-144) using a clinical audiometer (Grason-Stadler, model 61) with standard earphones (Telephonics, TDH-50P) enclosed in supra-aural ear cushions (MX-41/AR). Pure-tone air-conduction audiometry was performed at octave frequencies from 0.25 through 8 kHz and at one half-octave frequency (3 kHz). Audiometric measurements were made separately for each ear. Bone-conduction testing used a standard oscillator (Radioear, model B-71) and headband. Bone-conduction thresholds were assessed at octave frequencies from 0.25 through 4 kHz. However, it should be noted that if participants had audiological testing performed by a certified/state-licensed audiologist, including tests obtained from a Veteran's Administration Hospital within 3 months of recruitment/admission to this experiment, standard audiological testing was not repeated.

2.8. Psychophysical loudness scaling

Loudness testing was based on a computer-controlled magnitude-estimation procedure for a 1 kHz pure tone. Tone duration was set at 500 ms and individual stimuli were presented at 7

discrete SPLs (20, 30, 40, 50, 60, 70, 80 dB) with each tone being presented three times in random order. For this task, the individual was asked to provide a number between 1 and 100 that corresponded to the loudness level of the tone being presented and then was instructed to type that specific number into a box on the computer screen which was allocated for this operation. The onset and duration of the tone was synchronized to a visual white box in the center of the computer screen to indicate tone presentation and also as a cue to the participant. To put the magnitude estimation procedure in context, an anchored scale was utilized where individuals were instructed such that use of the number "1" was considered the softest sound one could imagine and the number "100" was considered the loudest sound one could possibly tolerate. Once this scaling procedure was completed, the individual was asked to estimate the loudness of their tinnitus from 1 to 100, in relation to the 1 kHz tone. Then, from the *y*-axis, that specific number was used to bisect that person's magnitude estimation function and find the loudness level on the *x*-axis of the function that corresponded to the individual's tinnitus (Fig. 2).

2.9. Repetitive transcranial magnetic stimulation (rTMS)

The intensity of the *r*TMS and side of stimulation was based on an approach used by Piccarillo et al. (2011) and others in the field. Repetitive TMS was generated by a commercial system (MAGSTIM *Rapid* device) with booster module *plus* (model: CE 0086) and active (model: P/N 1640–00) and sham (model: S/P 16080) figure-of-eight coils. Triggering of the MAGSTIM unit was accomplished by a transistor-transistor logic (TTL) pulse from a function generator

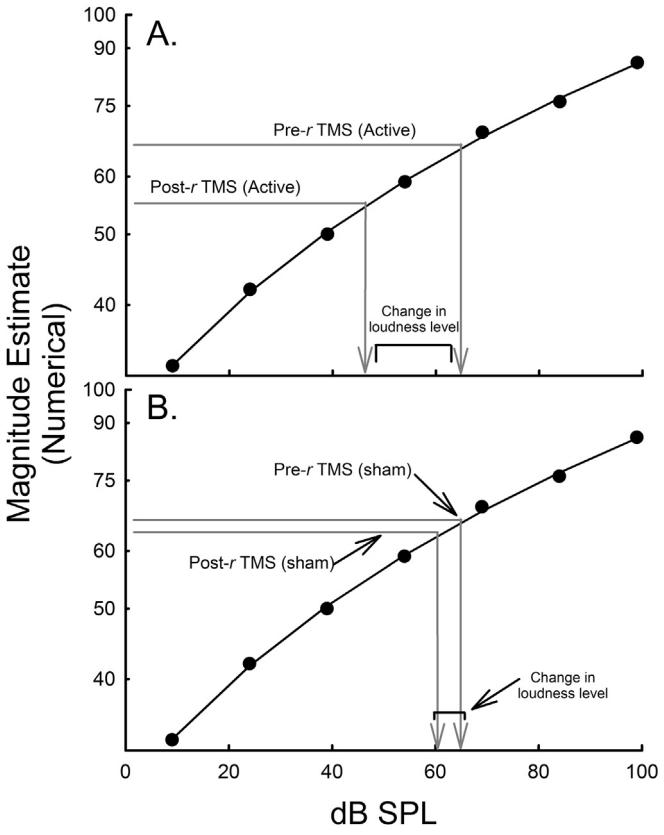

Fig. 2. Top A (Active). Magnitude estimation function where individuals estimate tinnitus loudness levels, pre and post *active* *r*TMS. **Bottom B (Sham)**. Magnitude estimation function where individuals estimate tinnitus loudness levels, pre and post *sham* *r*TMS.

(BK Precision model: 4003A) and the visual display of the trigger pulse and timing was monitored continuously on an analog oscilloscope (Hitachi model: V-212). The figure-of-eight coil was used because it produced a more focal activation in comparison to other coil types available at the time (Hallett, 2000). The figure-of-eight coils (sham and active) were attached to an adjustable multi-articulating boom stand to position the coils over the left side of the head.

The sham coil was designed to be identical in appearance to the active coil. However, as we noted above, prior to beginning this experiment, it was observed that at equivalent output settings on the MAGSTIM power unit, the sham coil produced noticeably higher acoustic output levels than the active coil. This observation was confirmed in a separate pilot study (Cacace et al., 2009) described in Appendix 1 and allowed us to equate the stimulus SPLs on a person-by-person basis.

The articulating boom arm of the stand allowed for the coil to be positioned over the temporal cortex of the left hemisphere T3/T5 (halfway between the standard 10–20 EEG coordinate system (T3 and T5) of the International Federation based on methods used by others (Langguth et al., 2006; Lee et al., 2008; Cacace et al., 1988), with the arm of the coil directed upward. Prior to initiating the experimental stimulation protocol, we obtained a motor threshold by stimulating the motor cortex so as to elicit a contraction in the right thumb abductor muscle. The *r*TMS stimulation was presented at a rate of 1 Hz and at a power setting of 110% above the motor threshold for each individual. The duration of each individual session was set at 20 min providing for a total of 1200 stimuli for that period-of-time. Participants received 5 sequential days of active and 5 sequential days of sham *r*TMS stimulation.

Additionally, prior to *r*TMS, participants were fitted with passive acoustic attenuators (foam insert ear plugs; 3M Classic™) for each ear and testing was performed in a dimly lit sound attenuating test booth described previously which accommodated both the examiner and examinee. Participants were seated in a comfortable chair and the figure-of-eight coil was positioned over the T3/T5 test site on the head. Participants, but not the experimenter, were blinded as to whether the stimulation condition was active or sham. Prior to *r*TMS (either active or sham), participants completed the THQ and a computer controlled magnitude estimation procedure to estimate the loudness level of their tinnitus. After *r*TMS treatment was completed, participants were taken to the MRI Research Facility at Harper Hospital, Detroit, MI, where MRI and MRS testing was performed. On the 5th sequential day following *r*TMS treatment, participants filled out another THQ, repeated the loudness measures and a second set of identical MRI measures were obtained. In addition and prior to each MRI examination, an MRI safety questionnaire was filled out to ensure that no changes occurred from the previous exam that might jeopardize subject safety within the scanner (Shellock, 2011). A registered nurse associated with the MRI Research Facility reviewed the safety questionnaire with every participant to ensure accuracy prior to scanning. After medical history and successful safety questionnaire administration/completion, the MRI assessment was performed.

3. Magnetic resonance imaging (MRI)

3.1. Procedure

Individual MRI scanning sessions typically took on the order of 45 min to complete. Each examination included a high resolution anatomical scan and MRS of the auditory cortex in the left and right temporal lobe. The magnetization-prepared rapid gradient-echo (MPRAGE) pulse sequence was used for acquiring high resolution anatomical images, which served to identify and place a 3-D voxel

to acquire data for subsequent MRS assessment. A specifically designed Stimulated Echo Acquisition Mode (STEAM) pulse sequence with the capability of resolving glutamate, glutamine and γ-aminobutyric (GABA) was applied (Yang et al., 2007; Hu et al., 2007). Fig. 3 provides a graphic visualization of the MRI/MRS outputs in diagrammatic form.

3.2. Instrumentation

Magnetic resonance imaging data were collected on a 3T (Tesla) Siemens MAGNETOM Verio scanner employing a 32-channel head coil. Briefly, the MPRAGE sequence is designed for rapid acquisition with T1 weighted anatomical MRI. Fast gradient echoes are characterized by their rapid sampling time, high signal intensity, and image contrast while approaching steady state. With appropriately chosen parameters, MPRAGE provides good contrast between gray matter, white matter, and cerebro-spinal fluid. The parameters used for our experiments are: number of slices = 176, field-of-view matrix = 256 mm × 256 mm, pixel size = 0.7 × 0.7 × 1.3 mm^3, TR = 1.6s, TE = 4.38, TI = 800 ms, FA = 8, total acquisition time = 5:26 min.

3.3. Magnetic resonance spectroscopy

The single voxel STEAM sequence was used for MRS data collection. A manual adjustment shimming process (frequency, transmitter, and water suppression) was always used prior to data collection. The parameters for MRS included: Voxel Volume = 15 mm × 20 mm x 15 mm, TR = 1500 ms, TE = 72 ms, TM = 6 ms, BW = 1200 Hz, AVE = 256, TA = 6'30", total acquisition time = 6:36 min. As a powerful extension of the high resolution MRI, ^{1}H MRS provides a biochemical assay of brain tissue at the molecular level. Conventional *in vivo* brain ^{1}H MRS can detect metabolites and neurotransmitters including: N-acetylaspartate (NAA), total choline (Cho), total creatine (Cr), myo-inositol (MI), lactic acid (Lac), lipid (Lip), as well as the mixed signal of glutamate, glutamine, and GABA, in some, but not all cases/conditions.

4. Analysis

4.1. Statistical analysis and data presentation

The analyses performed on each data set were based on difference values between the pre/post sham and pre/post active conditions over the 5 sequential days of treatment. For example, if the active pre *r*TMS value was 50 on the THQ and this was reduced to a score of 40 on the post activation condition, then the difference value would be 10. Based on this approach, a positive number indicates a *reduction or improvement* in response to that questionnaire category. Similarly, if an individual indicated that their tinnitus loudness level in the pre active condition was initially 60 dB and this value was changed to 45 dB following 5 sequential days of active stimulation, then this would be considered an improvement of 15 dB (i.e., a reduction in tinnitus loudness level); again, a positive number.

Pre/post differences for loudness levels and individual THQ categories for the sham and active *r*TMS conditions were evaluated with 2-tailed paired *t*-tests. For each of the metabolites studied, a 2-way condition (sham *vs*. active) by hemisphere (left *vs*. right) repeated measures analysis-of-variance (ANOVA) was performed. If significant interactions were observed, then *post-hoc* testing was performed. We also analyzed individual variables with a Pearson's Product Moment Correlation Analysis.

Analysis of MRS data was based on the LCModel (Provencher, 1993). Individual spectra were computed and numerical values were stored on an Excel spreadsheet.

5. Results

5.1. Safety-related considerations

None of the participants reported any adverse side effects resulting from *r*TMS over the course of 5 sequential days of either sham or active stimulation or from any of the four MRI/MRS scans that were performed. Therefore, with this repeated measures

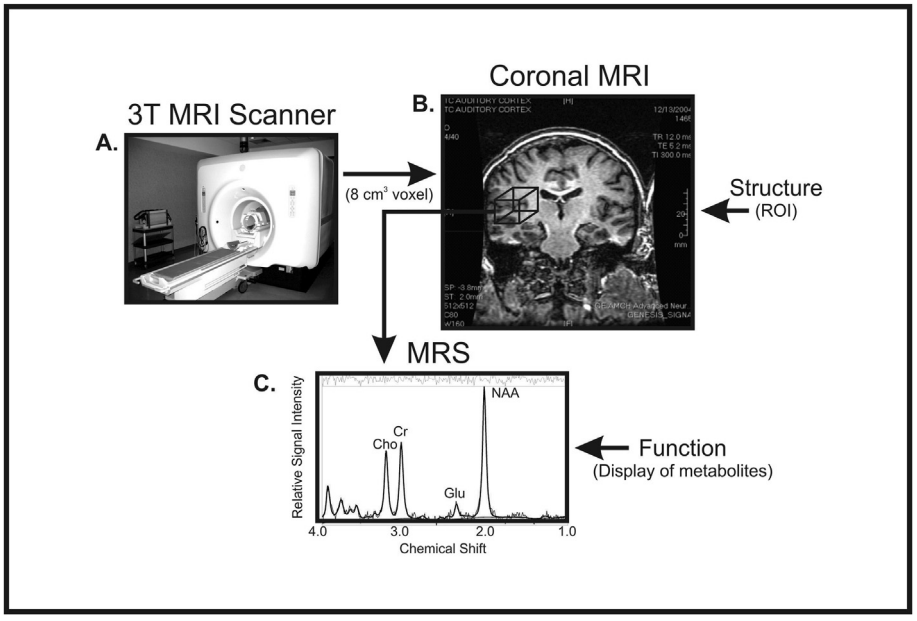

Fig. 3. Experimental setup for MRS. **Panel A (top left)** shows the 3T Siemens Verio MRI scanner; **Panel B (top right)** shows a coronal MRI slice of the brain with a single 3D voxel placed in the auditory cortical area of the left hemisphere; **Panel c (bottom)**, shows a sample spectra derived from auditory cortex depicting various metabolites quantified in this investigation using the LCModel. Abbreviations represent: *N*-Acetyl aspartate, NAA; Glutamate, Glu; Creatine, Cr; Choline, Cho.

design, inclusion criteria, and parameters utilized, the *r*TMS, MRI procedures, and psychoacoustic testing, were considered safe in our hands.

5.2. Pure tone audiometric data

Based on data averaged across all 25 participants for left and right ears, normal pure tone hearing sensitivity was observed in the 0.25–1 kHz range; mild to moderately severe pure tone hearing thresholds were observed in the 2–8 kHz range, bilaterally. Note that there was greater threshold variability in the higher frequencies for both right and left ears (Fig. 4), consistent with the etiology and age range of participants.

5.3. Loudness levels

Changes in loudness level for the individual participants are shown in Fig. 5 for each of the experimental conditions. In these data, *y*-axis indicates change in tinnitus loudness level in dB; *x*-axis indicates the two experiment conditions. Positive values of pre/post treatment differences represent a decrease in tinnitus loudness levels; negative values represent an increase in tinnitus loudness levels; and no difference is indicated by the dotted zero line. For the sham *r*TMS condition (filled black circles), the mean differences in tinnitus loudness level approximated 0.0 dB (horizontal black bar); for the active *r*TMS condition (filled gray circles), reduced tinnitus loudness levels were observed (mean difference approximated 4.5 dB (horizontal dark gray bar). Loudness level changes were significantly larger for active than for sham ($t = -3.45$, $p = 0.001$.

6. Tinnitus Handicap Questionnaire

6.1. Subscales

6.1.1. Social, Emotional, behavioral (SEB)

A significant reduction in the SEB score was observed following active *vs*. sham *r*TMS stimulation based on a paired *t*-test ($t = -2.60$, $p = 0.012$). The mean pre/post change was 4.37% (active group); *vs*. 0.58% (sham group).

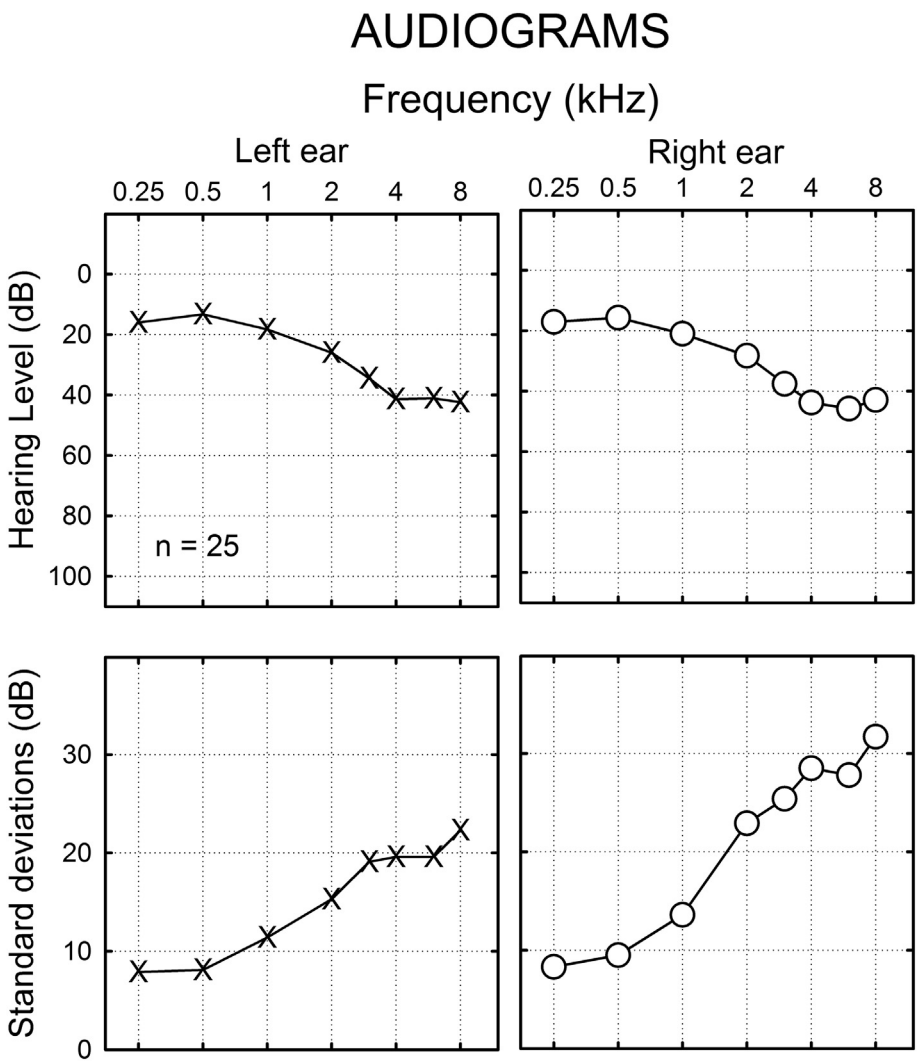

Fig. 4. Top) Average pure-tone thresholds for left and right ears at octave and half-octave frequencies over a bandwidth from 0.25 to 8 kHz. The average threshold levels range from borderline normal (0.25–0.5 kHz) to mild-to-moderate sensorineural hearing loss (1–8 kHz). Note that audiometric thresholds were relatively symmetric between the left and right ears. Bone-conduction thresholds, not shown, approximate air-conduction thresholds in all participants. **Bottom)** Standard deviation (SD) values corresponding to the same frequency range noted in the threshold plots. Better hearing thresholds correspond with lower SDs whereas worse thresholds correspond to higher SDs. Thus, taking variability into account, hearing thresholds range from normal hearing sensitivity in the low to mid frequencies to mild to moderately severe hearing loss in the higher frequency range.

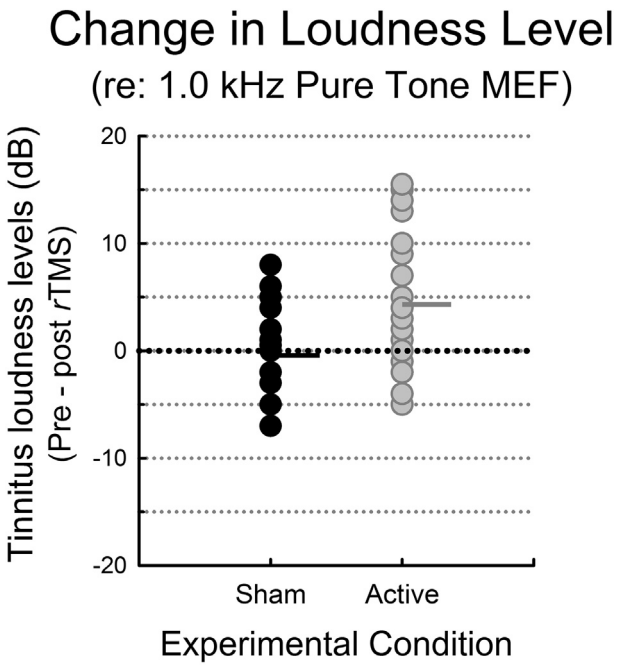

Fig. 5. Vertical scatter plot of pre and post differences in tinnitus loudness levels following sham *r*TMS (black filled circles) and active *r*TMS stimulations (gray filled circles) where, the y-axis represents difference values between pre/post sham and pre/post active conditions. The black and filled gray circles represent individual data points. Separate black and dark gray horizontal bars represent mean values. Positive values represent a *reduction* in loudness levels; negative values represent an increase in loudness levels. A significant reduction in loudness levels occurred in the active *vs.* sham stimulation conditions. Loudness data were obtained from individual magnitude estimation functions (MEFs).

6.1.2. Tinnitus and hearing (T&H)

A significant reduction in the T&H score was observed following active *r*TMS stimulation based on a paired *t*-test ($t = -4.16$, $p < 0.001$). The mean pre/post change in THQ T&H was 5.73% (active group); 0.0% (sham group).

6.1.3. Total score

A significant reduction was observed for the Total score following active *r*TMS stimulation based on a paired *t*-test ($t = -2.93$, $p = 0.005$). The mean pre/post change in the total score was 6.52%, (active group); and 1.36% (sham group).

7. Magnetic resonance spectroscopy

7.1. Metabolites

7.1.1. Choline

Based on a 2-way repeated measures ANOVA, there was neither a main effect of hemisphere ($F = 0.062$, $p > 0.78$) or condition ($F = 0.713$, $p = 0.4$); nor was there a significant hemisphere × condition interaction ($F = 1.55$, $p = 0.207$). These results are shown in Fig. 6.

7.1.2. N-acetyl aspartate (NAA)

Based on a 2-way repeated measures ANOVA, there was neither a main effect of hemisphere ($F = 0.286$, $p = 0.594$) or condition ($F = 0.010$, $p = 0.922$), nor was there a hemisphere × condition interaction ($F = 0.866$, $p = 0.217$). These effects are shown in Fig. 7.

7.1.3. Glutamate

While there was not a main effect of hemisphere ($F = 2.01$, $p > 0.15$), there was a significant main effect of condition ($F = 10.4$, $p = 0.002$), as well as a significant hemisphere × condition interaction ($F = 7.89$, $p = 0.006$). These data are shown in Fig. 8. *Post-hoc* testing evaluated all relevant conditions. Significant differences were observed between left hemisphere (active condition) *vs.* left hemisphere (sham condition; $p < 0.004$); left hemisphere active *vs.* right hemisphere sham ($p < 0.008$) or right hemisphere active condition; $p < 0.02$) conditions. These results indicate that glutamate levels were reduced in the left hemisphere but only for the active stimulation condition. Most notably, changes in glutamate concentrations were *not* observed in the right (unstimulated) hemisphere. These data were independent of all pre-experimental baseline values used in the computations; only Glu combinations are shown (Fig. 9).

It is also important to note that while we were very interested in studying GABA, these measures within left and right auditory cortical areas were not detectable for the majority of participants studied and could not be analyzed for purposes of this investigation. In the context of the LCModel, this occurs when the Cramér-Rao lower bounds variance of the expected metabolite spectra

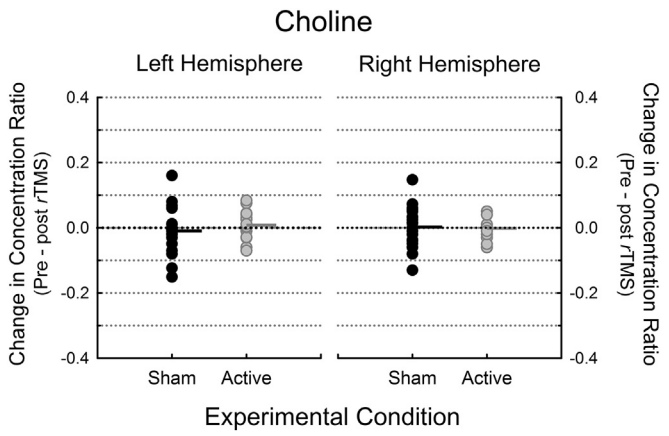

Fig. 6. Vertical scatter plot of pre/post differences in Cho levels following sham *r*TMS (black filled circles) and active *r*TMS (gray filled circles) shown separately for left and right hemispheres. The *x-axis* represents the experimental conditions; the *y-axis* represents the ratio of Cho to Cr. Horizontal bars represent mean values. Main effects and interactions of the repeated measures ANOVA were not significant ($P > 0.05$).

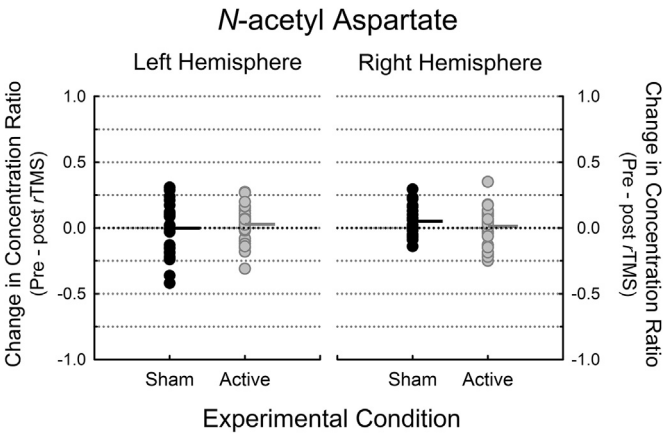

Fig. 7. Vertical scatter plot of pre/post differences in NAA levels following sham *r*TMS (black filled circles) and active *r*TMS (gray filled circles) for left and right hemispheres. The *x-axis* represents the experimental conditions; the *y-axis* represents the ratio of NAA to Cr. Horizontal bars represent mean values. Main effects and interactions of the repeated measures ANOVA were not significant ($P > 0.05$).

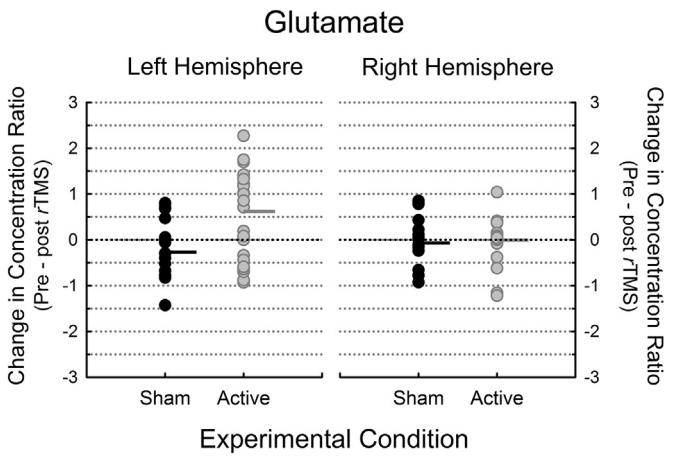

Fig. 8. Vertical scatter plot of pre/post differences in Glu levels following sham *r*TMS (black filled circles) and active *r*TMS (gray filled circles) for left and right hemispheres. The *x-axis* represents the experimental conditions; the *y-axis* represents the ratio of Glu to Cr. Horizontal bars represent mean values. A significant reduction in Glu was found over the left hemisphere and only in the active *r*TMS condition.

exceeds 25% (Provencher, 1993).

7.2. Pearson Product Moment Correlation Analyses

We also evaluated the relationships among all variables and experimental conditions by Pearson Product Moment Correlation Analyses (Pearson's *r*). From this matrix of variables, significant pair-wise correlations were observed among THQ variables, THQ-MRS metabolite variables, metabolite-metabolite variables, and metabolite-loudness variables. Significant pair-wise correlations were found for: THQ SEB active *vs*. THQ total active, $r = 0.61$, $p < 0.05$; THQ T&H sham *vs*. THQ total sham, $r = 0.59$, $p < 0.05$; and THQ SEB sham *vs*. THQ Total sham, $r = 0.49$, $p < 0.05$).

There were significant pair-wise correlations among THQ-metabolite variables: (Glut RH active *vs*. THQ T&H active, $r = 0.47$, $p < 0.05$; Cho RH active *vs*. THQ LH SEB sham, $r = -0.46$, $p < 0.05$), although outliers may have influenced these results.

There were significant pair-wise correlations among metabolite-metabolite variables (NAA left hemisphere active *vs*. Cho right hemisphere sham, $r = 0.44$, $p < 0.05$; NAA right hemisphere sham *vs*. NAA right hemisphere active, $r = 0.49$, $p < 0.05$).

Lastly, there was a significant pair-wise correlation between the metabolite-loudness variables; specifically noteworthy was the reduction in tinnitus loudness level that was positively correlated with a down regulation of Glu. The importance of this finding was that it only occurred in the active *r*TMS condition and that it was specific to the hemisphere that was stimulated ($r = 0.77$, $p < 0.05$; Fig. 10).

8. Discussion

We used a prospective randomized single-blinded sham-

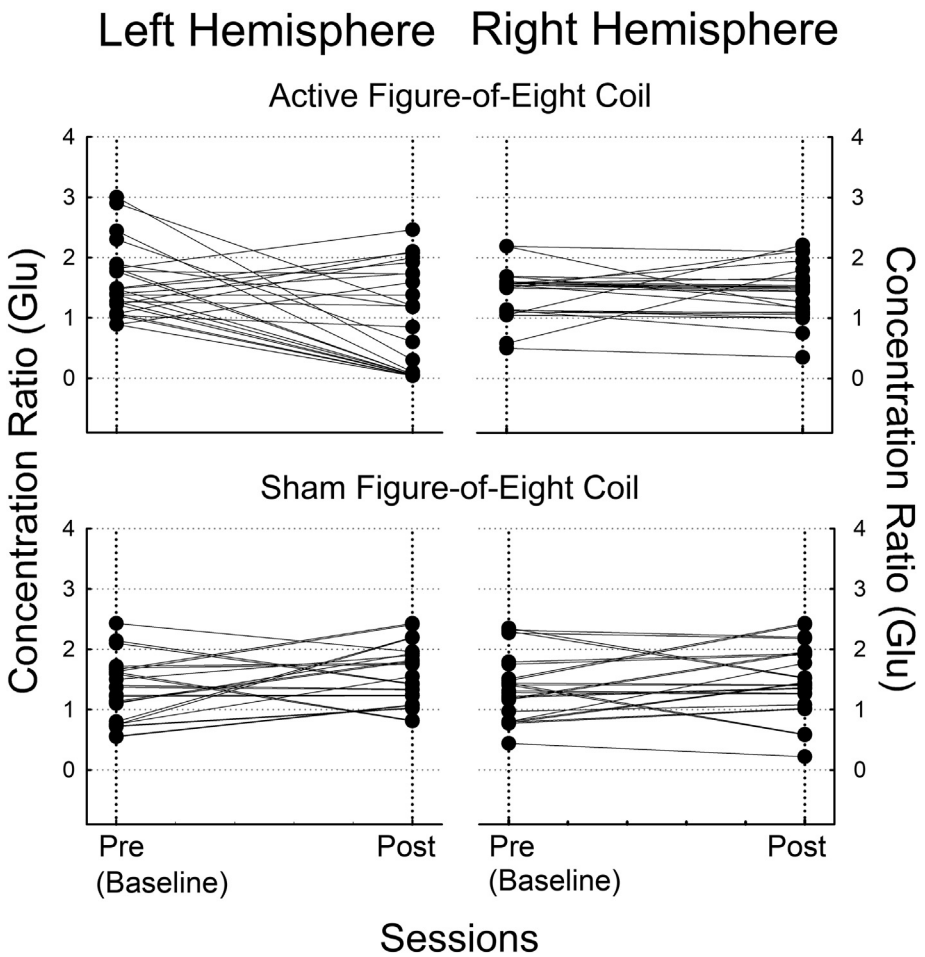

Fig. 9. Pre/post Glu baseline (pre) and post *r*TMS levels for active (top) and sham *r*TMS (bottom) *r*TMS conditions recorded from left and right hemispheres, are shown respectively. The trends seen in these data (increases or decreases) capture the difference values that are plotted in Fig. 8.

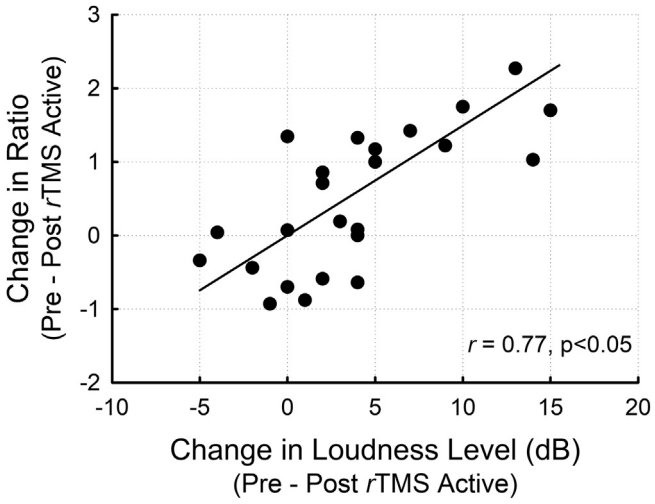

Fig. 10. Scatter/regression plot showing the significant pair-wise correlation ($r = 0.77$, $p < 0.05$) between a down regulation in Glu concentrations (*y*-axis) *vs*. the reduction in tinnitus loudness levels following active *r*TMS (*x*-axis).

controlled cross-over design to study the effectiveness of *r*TMS in suppressing noise-induced tinnitus. This is one of the first studies to our knowledge which evaluated functional relationships among neurochemical outcome measures in humans obtained non-invasively from auditory cortical areas of the left and right temporal lobe using MRS, direct psychophysical loudness measures using a magnitude estimation procedure, and self-perceived changes using a standardized tinnitus questionnaire (THQ). We also show that in our hands, the methodologies utilized were safe and there were no adverse side effects reported for either *r*TMS, MRI/MRS, or psychoacoustic testing.

As far as MRS was concerned, we did not find any significant changes in Cho or NAA following *r*TMS. The most prominent changes observed were reductions in Glu levels which were specific to auditory cortical areas of the left hemisphere and only for the active *r*TMS condition. Most notably, this reduction in Glu was correlated with a reduction in tinnitus loudness levels. The correlation value was significant and strong ($r = 0.77$, $p < 0.05$).

Currently, there are two experiments that have used MRS to study mechanisms of tinnitus: one *ex vivo* study based on an animal model using Long Evans rats following noise exposure (Brozoski et al., 2012) and another comparing two groups of humans with and without tinnitus matched for age, gender, and hearing loss (Sedley et al., 2015). Based on the *ex vivo* MRS measures in rats with behavioral evidence of tinnitus, Brozoski et al. (2012) found that Glu was increased bilaterally in dorsal cochlear nucleus and primary auditory cortex and that GABA levels were reduced in the medial geniculate body. No differences in Glu or GABA were observed in the inferior colliculus. Thus, the Brozoski et al. (2012) study was noteworthy in showing region specific changes in inhibition and excitation at different levels in afferent auditory pathways. Sedley et al. (2015) used the MEGA PRESS pulse sequence (MEshcher–GArwood Point RESolved Spectroscopy) to detect GABA levels in auditory cortex, which was the main impetus for their study. However, interpreting MRS data from edited GABA pulse sequences is under refinement and still evolving (see Mullins et al., 2014 for a review). It may be the case that higher field strength MRI scanners (i.e., >3.0T) may be necessary to detect GABA concentration reliably. However, this remains to be determined. Nevertheless, Sedley et al. (2015) found an asymmetry (reduction) in GABA concentrations in their tinnitus group, most notable in right *vs*. left auditory cortex. Interestingly, severity of tinnitus, which was based on a subjective 10 point rating scale and the degree of hearing loss, positively correlated with choline levels.

In terms of theory, the biochemical hypothesis associated with tinnitus generation is consistent with converging evidence that partial or complete peripheral deafferentation from acoustic trauma (hearing loss associated with hair cell damage and auditory nerve degeneration) results in a cascade of changes in the auditory periphery and central nervous system (CNS) including reduced afferent input to the CNS that induces an imbalance between inhibitory and excitatory inputs to auditory neurons at various levels in central auditory pathways (e.g., Middleton et al., 2011; Wang et al., 2011; Yang et al., 2011; Godfrey et al., 2012; Rüttiger et al., 2013; Lee and Godfrey, 2015). This framework suggests that changes in inhibitory and excitatory equilibrium destabilizes circuits in the brainstem and cortex resulting in plastic readjustments attempting to compensate for this disparity, so-called homeostatic plasticity (e.g., Eggermont and Roberts, 2004; Schaette and Kempter, 2006; Roberts et al., 2010). Thus, either alone or in combination, neuronal hyperactivity, bursting discharges, and increased cortical or brainstem/diencephalic neural synchrony are thought to be mechanisms associated with pathologic plasticity that result in the perception of tinnitus (e.g., Dong et al., 2010; Henderson et al., 2011; Kaltenbach, 2011; Brozoski et al., 2012). Thus, research indicates that following noise induced hearing loss, biochemical mechanisms characterized by a down regulation of inhibitory (GABAergic) neuronal input and/or an up regulation in excitatory (glutamatergic) input to neurons in areas such as cochlear nucleus, superior olivary complex, inferior colliculus and possibly, auditory cortex can occur (see Kaltenbach, 2011 for a review). Indeed, based on the chronology of advancements in tinnitus research over the last 3 decades, it is reasonable to suggest that one of the most prominent hypotheses of tinnitus generation at the cellular level subsumes a biochemical basis. Consequently, MRS is an essential and logical approach to understand tinnitus and to potentially monitor pharmacologic, electric, and/or magnetic treatments. Because of time limitations and considerations for obtaining a favorable signal-to-noise ratio, our MRS testing was limited to a single voxel placement within left and right auditory cortical areas. Accumulating evidence from studies in rat indicates that ^{1}H-MRS can identify and separate excitatory and inhibitory compounds *in vivo* when appropriate pulse sequence and magnetic strength parameters are utilized (e.g., Hu et al., 2007; Yang et al., 2007). Unfortunately, in the current study, GABA was not detectable in the majority of individuals. As we mentioned previously, it is possible that with higher field strength MRI scanners (i.e., 7.0T), detection of GABA levels in auditory cortex may be achievable on a more reliable basis. But this will have to await future investigations. However, in the current study, the Glu signal was robust in all individuals and the reduction in this metabolite was noteworthy since it corresponded with a significant reduction in tinnitus loudness levels. Interestingly, significant but not large self-perceived changes in the tinnitus perception were also noted on the THQ (SEB, T&H, Total scores) and these improvements only occurred in the active *r*TMS condition.

It is also of interest to note that much higher resolution MRS paradigms are available to basic science researchers that have already provided advancements and established relationships between magic-angle spinning MRS and cognitive variables following traumatic brain injury (TBI) from shock tube related blast exposure in rats (e.g., Sajja et al., 2012, 2013; 2014). While these recent experiments were designed specifically to evaluate TBI following controlled blast overpressures, studying tinnitus with this methodology was not performed but represents a logical approach that can be used in future investigations.

In summary, this study represents a first step in establishing

perceptual, neurochemical, and questionnaire-based relationships in a non-invasive manner when applied to noise-induced tinnitus research in humans using an *r*TMS framework. Consequently, it sets the stage for other studies that can build on these relationships and enhance treatment modalities for tinnitus. Although *r*TMS is non-invasive and lacks substantial risks, it can be potentially confounded by acoustic stimulation of the activating coils both in the sham and active stimulation conditions (see Appendix 1). While the primary biochemical, psychoacoustic, and questionnaire-based effects we observed only occurred in the *active* condition over the left temporal lobe, our data argues convincingly that the *r*TMS was the main effect accounting for our results. Nevertheless, while acoustic artifacts generated from *r*TMS coils have been ignored in the majority of tinnitus related *r*TMS studies, this area needs to be addressed and clarified more thoroughly in future investigations.

Acknowledgements

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Appendix 1

Acoustical transients with levels as high as 130 peak SPL (pSPL) have been reported when magnetic coils are activated during single or *r*TMS experiments. This observation led early investigators to be concerned about inducing temporary or permanent threshold shifts in participants under various experimental conditions (e.g., Counter and Borg, 1992, 1993; Counter et al., 1991, 1993; Counter, 1993). In a somewhat different context, we informally observed that the "sham" figure-of-eight coil used in this experiment, seemed by subjective accounts to produce a much higher SPL output when compared to the "active" figure-of-eight coil at equivalent power settings. Because of the design features of this experiment (sham-controlled cross-over) and the necessity to control for as many factors as possible to avoid confounding, this prompted us to perform a small pilot study to investigate the acoustic output characteristics of the sham *vs*. active figure-of-eight coils. If our informal observations were indeed true, then this would allow us to control for intensity differences between coils by measuring individual input-output characteristics and also allow us to investigate time and frequency domains factors.

Methods

The acoustic measurements obtained herein were generated by the same commercial system used in the current experiment. Sound field acoustic measurements were made using a sound-level meter (SLM; Brüel & Kjær, model 2209) in peak-hold mode with a 1" condenser microphone (Brüel & Kjær, model 4145). Acoustic measurements from the ear canal were made using a commercially-available probe-microphone system (Etymotic, ER-7C) with the probe tube positioned near the lateral surface of the tympanic membrane. To quantify the sound field probe microphone system, instrumental outputs were routed to a digital oscilloscope/spectrum analyzer (PICO Electronics, model 2203) via a universal serial bus (USB) to a laptop computer (Dell XPS, model 1530) where data were stored for offline analysis. A block diagram and graphic representation is shown in Fig. 11.

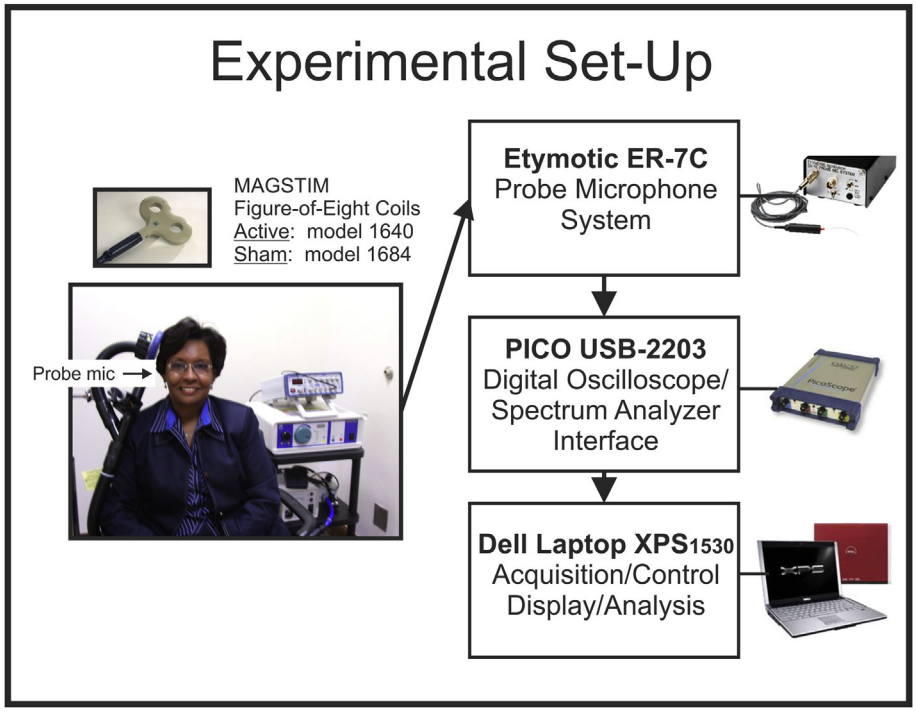

Fig. 11. Block diagram of the experimental set-up used to measure SPLs at the lateral surface of the tympanic membrane in the ear canal with a probe-microphone system in place.

Input-output functions for the sham and active coils were made across a broad range of power output levels of the MAGSTIM device (10–100%; in 10% increments and at a distance of ~1 m) using the SLM described above. The rationale for using the distance of 1 m from the SLM to the active figure-of-eight coil, was to avoid damaging the highly sensitive B&K condenser microphone from the magnetic field of the active figure-of-eight coil. Input-output functions for sham and active coils are shown in Fig. 12 (top). The average difference between active and sham figure-of-eight coils was 25.5 dB (Fig. 12, bottom). The time domain waveforms and power spectra of the sham and active figure-of-eight coils are shown in Fig. 13; sham (top); active (bottom).

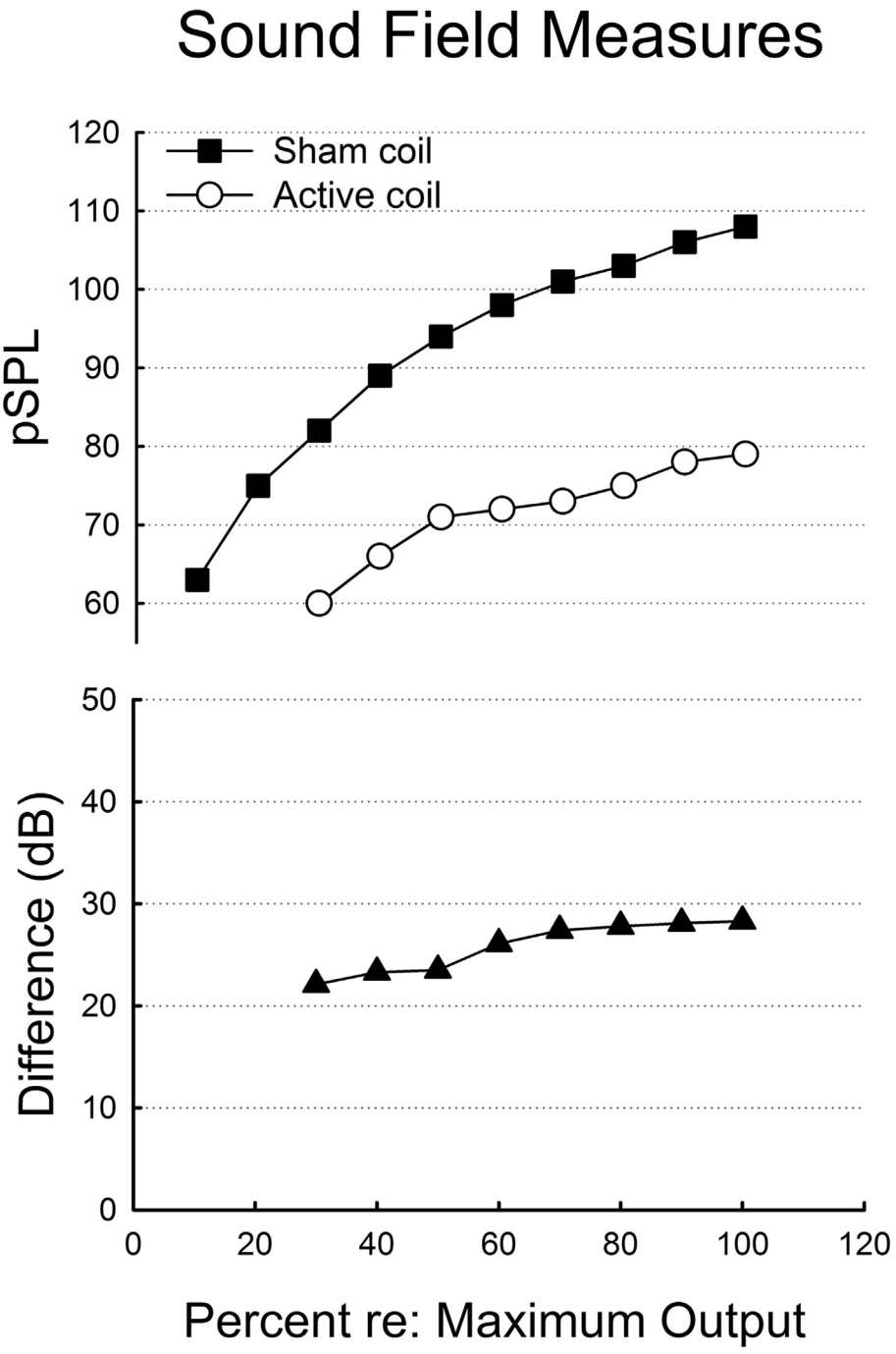

Fig. 12. Top) Input/output functions of sound field acoustic measures of sham and active figure-of-eight coils, where the *y*-axis represent peak sound pressure level (pSPL) and the *x*-axis represents maximum power output of the MAGSTIM commercial TMS device. It can be seen that the sham coil (filled squares) has much higher pSPL values than the active coil (unfilled circles) over a broad range of output values. The input-output functions were non-linear. **Bottom)** The average difference between the sham and active coils, as a function of maximum output level, was ~25.5 dB.

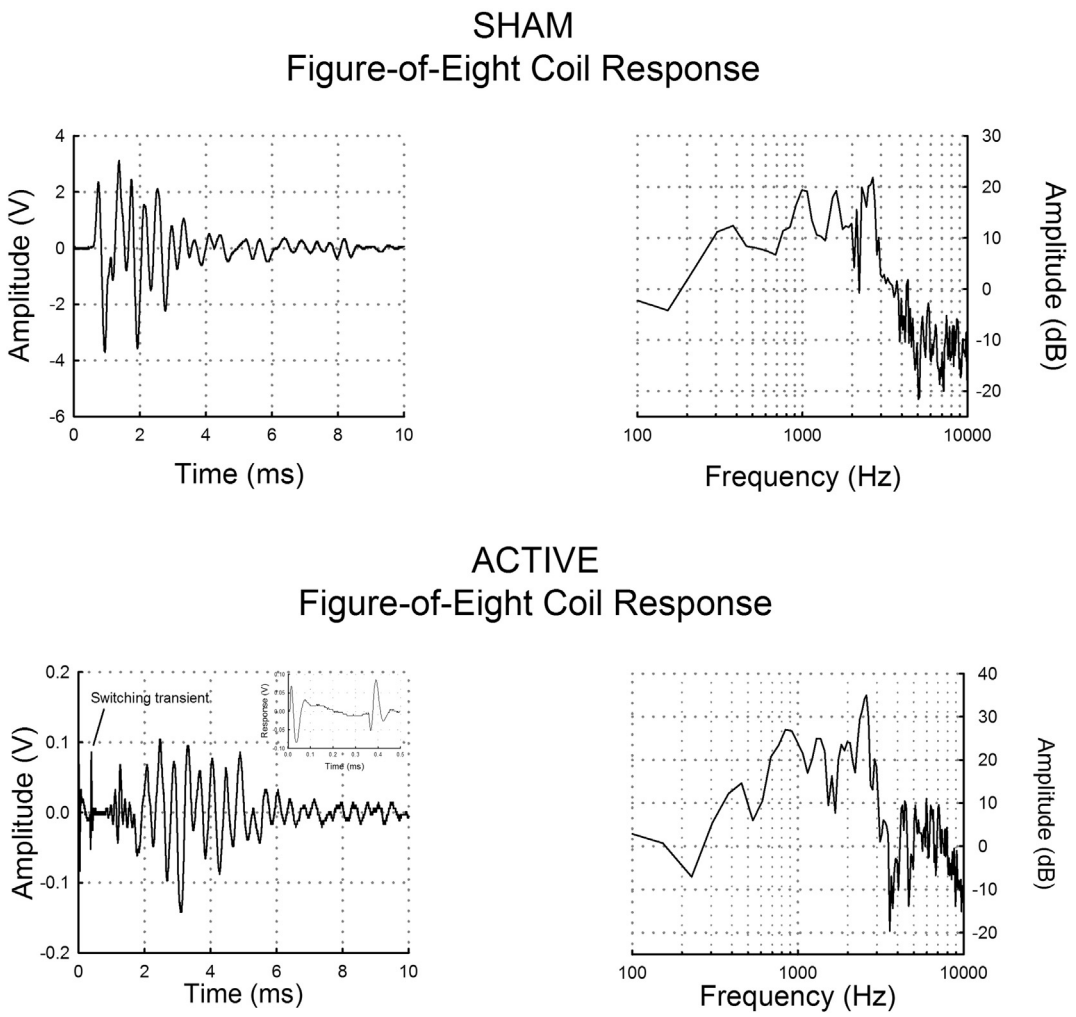

Fig. 13. Top left) Time domain waveform of sham figure-of-eight coil; **Top Right**) frequency domain power spectra of the sham figure-of-eight coil. **Bottom left**) Time domain waveform of the active figure-of-eight coil. The inserted small window shows an expanded time domain waveform of the switching transient at the beginning of the response. **Bottom Right**) frequency domain power spectra of the active figure-of-eight coil. These measures were taken from a probe microphone located near the lateral surface of the tympanic membrane in the ear canal.

Probe-microphone measurements using the sham coil were made at two discrete output levels (50% and 25% re: maximum output of the MAGSTIM device) and under three experimental conditions: 1) ear canal open (unobstructed), 2) ear canal with a commercially-available acoustic-attenuating insert foam earplug in place (3M Classic™) and 3) ear canal with an insert foam earplug in place plus use of circumaural earmuffs (EAR® Model 1000) covering the external ear and pinna. The foam earplugs were chosen for their comfort and noise-reduction ratings (NNRs); the earmuffs were chosen for their light-weight non-conductive plastic construction and comfortable fit, as characterized by foam-filled cushions with a pivoting ear-cup design and also for their NNRs.[1] All measurements were made in a commercial sound attenuating test booth (Acoustic Systems, model 144S).

[1] Foam ear plugs have a NRR of 29 dB, based on the rating standard used in the United States; circumaural earmuffs have a NRR of 20 dB.

Results

Our measurements confirmed that the sham figure-of-eight coil had much higher sound-field pSPLs than the active coil at equivalent output levels (Fig. 10A) and that the growth characteristics were nonlinear over the range utilized; with the average difference between the two coils approximating 25.5 dB (Fig. 12). The temporal (left) and spectral characteristics (right) of the sham (top) and active coils (bottom) at the 50% power setting measured by the probe microphone in the unoccluded ear canal condition is shown in Fig. 13 (top and bottom). Both coils had similar frequency domain characteristics which encompassed a bandwidth from ~0.1 to >6 kHz. Also note that inserted at the top portion of the time domain waveform (active coil, bottom), is an expanded representation (x-axis; 0.0–0.5 ms) of the onset and offset characteristics of the impulse transient in the time domain. While the activating electrical pulse duration underlying the click sound approximated ~450 μs, it can be seen in Fig. 13 that there is substantial ringing in the time domain after pulse offset. The ringing characteristics (duration of the response) were quantified by determining the peak value in the time domain and then taking the points 90% points down from this value and measuring the duration of the response on the x-axis. With the probe microphone in place, these measures

were also made for the sham condition and for the male and female volunteers respectively, as a function of the three measurement conditions (Fig. 14, males left side; females right side) for the three measurement conditions (open ear canal, with foam ear plug, and with foam ear plug plus ear muff). As shown, the ringing had the longest duration at the highest output level used (50%) and in the open (unoccluded) condition. These ringing effects were reduced in duration when passive attenuation devices were in place with the lowest effects occurring when the combination of insert foam earplug and the ear muffs were utilized.

The pSPLs for the male and female participants for the sham condition are shown in Fig. 15 (males, left side; females, right side) for the three measurement conditions (open ear canal, with foam ear plug, and with foam ear plug plus ear muff). Highest pSPLs are observed in the open/unoccluded condition with a progressive decrease in SPL when inserts and inserts plus ear-muffs are utilized. Females had the highest pSPLs observed.

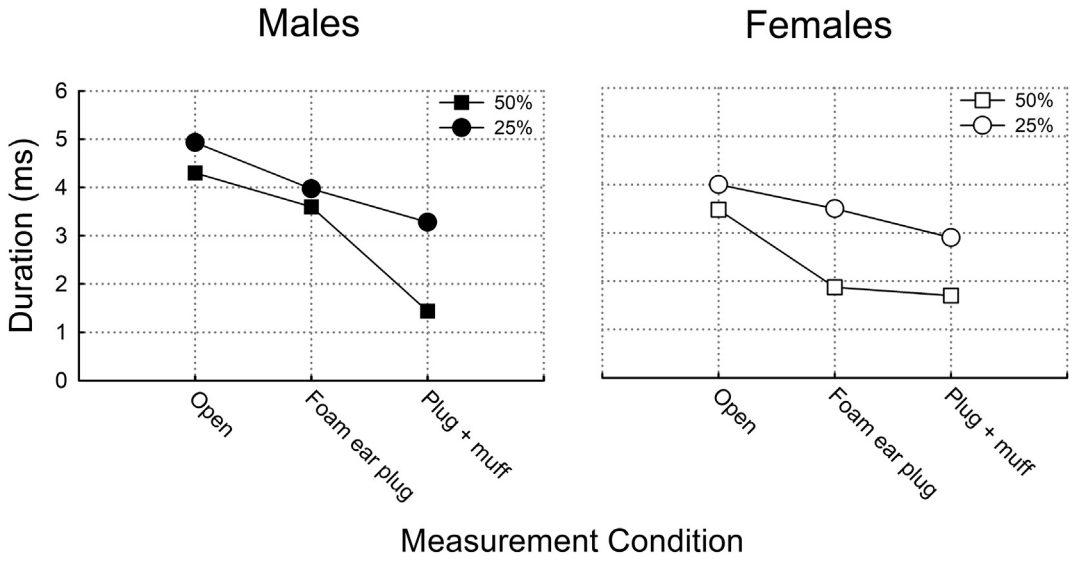

Fig. 14. The ringing characteristics (duration of the response) of the figure-of-eight coil from the probe microphone were quantified by determining the peak value in the time domain and then taking the points 90% points down from this value and measuring the duration of the response on the x-axis. With the probe microphone in place, these measures were also made for the sham condition and for the male and female volunteers respectively, as a function of the three measurement conditions (males left side; females right side) for the three measurement conditions (open ear canal, with foam ear plug, and with foam ear plug plus ear muff). The parameter of the graph is the power output of the MAGSTIM device set at 25% and 50% of maximum power output.

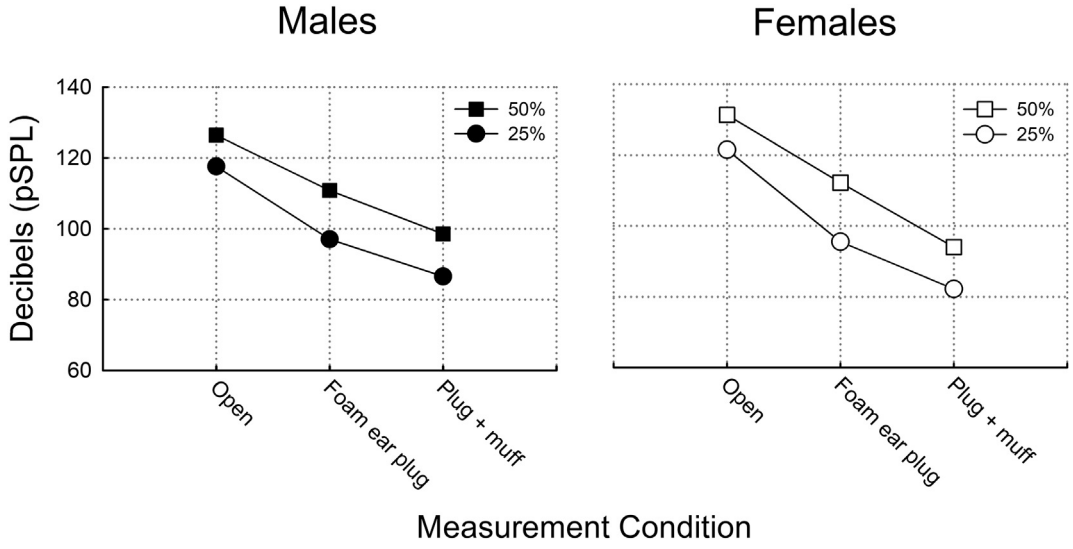

Fig. 15. Peak SPL from a commercially available probe-microphone assembly (Etymotic ER-7C) placed in the ear canal adjacent to the lateral surface of the tympanic membrane. Measures were obtained from the sham figure-of-eight coil in the time domain for adult female (n = 4) and male (n = 4) volunteers as a function of three measurement conditions; ear-canal open, ear-canal plugged with a foam acoustic attenuator (ear plug; 3M Classic™), and ear-canal plugged with a foam ear plug with the pinna covered with a commercial circumaural ear muff (EAR® Model 1000). The parameter of the graph is the power output of the MAGSTIM device set at 25% and 50% of maximum power output.

Discussion

Because at present, one cannot eliminate acoustic click sounds generated by the sham and active coils in these types of experiments particularly with respect to the equipment specified herein, knowing the input-output characteristics can allow investigators to adjust sham acoustic output level to be relatively equivalent to the active coil and for the most part, be indistinguishable to the individual under investigation. This is an important experimental design consideration to help control for confounding. Also note that the acoustic output plotted against dial setting is not a straight-line (Fig. 12, top) so that generating complete input-output functions is an important factor in order to closely match the output of the sham coil to that of the active coil. Ringing characteristic of the sham and active coils for individuals are shown in Fig. 13 and for each of the experimental condition for males and females (Fig. 14). Exactly how this response feature of the instrumentation might contribute to tinnitus-related studies is unknown. However, it should be noted that similar ringing effects were clearly observed in the seminal work of Counter and Borg (1992). The intensity characteristics for each of the experimental conditions are shown in Fig. 15. As might be expected, females had higher pSPL levels than males due to smaller ear canal volumes.

Other investigators have also recognized the importance of these acoustic contaminants in *r*TMS studies. Using a MagPro X100 with the MagOption *r*TMS system (Medtronics), Tringali et al. (2012) found that the active coil associated with this device produced “higher” output SPLs than the sham coil. While these findings are not consistent with the results found herein, they do agree in as much as the sham and active coils were not acoustically matched, and they speak to the importance that individual laboratories and investigators need to make independent acoustic measurements on their own instrumentation and these measures may differ depending on the manufacturer. For example, Dhamne et al. (2014) provided a comprehensive account of the output characteristics of several commercially available TMS devices. However, in contrast to the current study, Dhamne and colleagues only tested “active coils” and failed to characterize the temporal or spectral characteristics to any extent. For those interested in using *r*TMS in the context of sham controlled cross over designs that might involve auditory phenomena like tinnitus, the information contained in their paper is valuable but limited in scope.

It is also important to point out that if airborne acoustic artifacts are present during *r*TMS protocols, particularly with respect to tinnitus-related studies, they can complicate the interpretation of the results. This is highly relevant to issues related to ensure that tinnitus suppression is due predominantly to the magnetic stimulation and not from acoustically-driven radiations that may have alternative suppressive effects, such as “residual inhibition (RI)” (e.g., Feldman, 1971; Vernon et al., 1980; Tyler, 1982; Tyler et al., 1984; Roberts, 2007), and/or other transient or long-term plastic anatomical/structural or neurobiochemical changes that might occur in the brain (Tyler, 1984; Roberts, 2007; May et al., 2007). Recent engineering solutions in coil design demonstrate significant sound reduction methods in a proof-of-concept prototype device developed to address issues associated with acoustic artifacts during magnetic pulse activation (Peterchev et al., 2015). Lastly, possible effects of coil-skull interactions may also be present. In other words, in addition to airborne acoustic stimulation of the auditory pathways, bone-conduction stimulation is potentially a viable and alternative route when the coil is in contact with the skull. The effects of bone conduction were not assessed in the current pilot study, but should be considered in future investigations.

Conclusion

The acoustic output of sham and active figure-of-eight coils were found to be notably different in this investigation. Characterizing these effects can help to avoid/minimize confounding in sham-controlled cross-over designs. It is also clear that passive attenuation devices are effective in reducing, but not eliminating, airborne acoustic energy. At this point-in-time, the effects of coil ringing and bone-conducted vibrations are indeterminate and will need to be considered in future *r*TMS studies for tinnitus abatement.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.heares.2017.10.017.

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