Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Results Article - JoVE Produced Video
Manuscript Number:	JoVE63005R2
Full Title:	A pilot study on the repetitive transcranial magnetic stimulation of Aβ and tau levels in rhesus monkey cerebrospinal fluid
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TITLE:

A Pilot Study on the Repetitive Transcranial Magnetic Stimulation of Aβ and Tau Levels in Rhesus Monkey Cerebrospinal Fluid

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SUMMARY:

Here, we describe the procedure for a pilot study to explore the effect of repetitive transcranial magnetic stimulation with different frequencies (1 Hz/20 Hz/40 Hz) on Aβ and tau metabolism in rhesus monkey cerebrospinal fluid.

ABSTRACT:

Previous studies have demonstrated that a non-invasive light-flickering regime and auditory tone stimulation could affect Aβ and tau metabolism in the brain. As a non-invasive technique, repetitive transcranial magnetic stimulation (rTMS) has been applied for the treatment of neurodegenerative disorders. This study explored the effects of rTMS on Aβ and tau levels in rhesus monkey cerebrospinal fluid (CSF). This is a single-blind, self-controlled study. Three

different frequencies (low frequency, 1 Hz; high frequencies, 20 Hz and 40 Hz) of rTMS were used to stimulate the bilateral-dorsolateral prefrontal cortex (DLPFC) of the rhesus monkey. A catheterization method was used to collect CSF. All samples were subjected to liquid chip detection to analyze CSF biomarkers ($A\beta_{42}$, $A\beta_{42}/A\beta_{40}$, tTau, pTau). CSF biomarker levels changed with time after stimulation by rTMS. After stimulation, the $A\beta_{42}$ level in CSF showed an upward trend at all frequencies (1 Hz, 20 Hz, and 40 Hz), with more significant differences for the high-frequencies ($p < 0.05$) than for the low frequency.

After high-frequency rTMS, the total Tau (tTau) level of CSF immediately increased at the post-rTMS timepoint ($p < 0.05$) and gradually decreased by 24 h. Moreover, the results showed that the level of phosphorylated Tau (pTau) increased immediately after 40 Hz rTMS ($p < 0.05$). The ratio of $A\beta_{42}/A\beta_{40}$ showed an upward trend at 1 Hz and 20 Hz ($p < 0.05$). There was no significant difference in the tau levels with low-frequency (1 Hz) stimulation. Thus, high-frequencies (20 Hz and 40 Hz) of rTMS may have positive effects on Aβ and tau levels in rhesus monkey CSF, while low-frequency (1 Hz) rTMS can only affect Aβ levels.

INTRODUCTION:

Amyloid-β (Aβ) and tau are important CSF biomarkers. Aβ consists of 42 amino acids ($A\beta_{1-42}$), which is the product of transmembrane amyloid precursor protein (APP) hydrolyzed by β- and γ-secretases[1]. $A\beta_{1-42}$ may aggregate into extracellular amyloid plaques in the brain because of its solubility characteristics[1,2]. Tau is a microtubule-associated protein that is mainly present in axons and is involved in anterograde axonal transport[3]. Abnormal tau hyperphosphorylation is mainly induced by the imbalance between kinases and phosphatases, resulting in the detachment of tau from microtubules and the formation of neurofibrillary tangles (NFT)[1]. The concentration of tau increases in the CSF because tau and phosphorylated tau proteins (pTau) are released into the extracellular space during the neurodegenerative process. Previous studies have shown that CSF biomarkers are relevant to the three main pathological changes of the Alzheimer's disease (AD) brain: extracellular amyloid plaques, intracellular NFT formation, and neuron loss[4]. Abnormal concentrations of Aβ and tau present in the early stage of AD, thus allowing early AD diagnosis[5,6].

In 2016, Tsai et al. found that non-invasive light-flickering (40 Hz) reduced the levels of $A\beta_{1-40}$ and $A\beta_{1-42}$ in the visual cortex of pre-depositing mice[7]. Recently, they further reported that auditory tone stimulation (40 Hz) improved recognition and spatial memory, reduced amyloid protein levels in the hippocampus and auditory cortex (AC) of 5XFAD mice, and decreased pTau concentrations in the P301S tauopathy model[8]. These results indicate that non-invasive techniques could impact Aβ and tau metabolism.

As a non-invasive tool, transcranial magnetic stimulation (TMS) could electrically stimulate neural tissue, including the spinal cord, peripheral nerves, and cerebral cortex[9]. Moreover, it can modify the excitability of the cerebral cortex at the stimulated site and in the functional connections. Therefore, TMS has been used in the treatment of neurodegenerative disorders and prognostic and diagnostic tests. The most common form of clinical intervention in TMS, rTMS, can induce cortex activation, modify the excitability of the cortex, and regulate cognitive/motor function.

It was reported that 20 Hz rTMS had an *in vitro* neuroprotective effect against oxidative stressors, including glutamate and Aβ and improved the overall viability of monoclonal hippocampal HT22 cells in mice[10]. After 1 Hz rTMS stimulation, the β-site APP-cleaving enzyme 1, APP, and its C-terminal fragments in the hippocampus were considerably reduced. Notably, the impairment of long-term potentiation, spatial learning, and memory in hippocampal CA1 was reversed[11,12]. Bai et al. investigated the effect of rTMS on the Aβ-induced gamma oscillation dysfunction during a working memory test. They concluded that rTMS could reverse Aβ-induced dysfunction, resulting in potential benefits for working memory[13]. However, there are few reports on the effects of rTMS on tau metabolism and the dynamic changes in Aβ and tau in CSF before and after rTMS. This protocol describes the procedure for investigating the effects of rTMS at different frequencies (low frequency, 1 Hz; high frequencies,20 Hz, and 40 Hz) on Aβ and tau levels in rhesus monkey CSF.

PROTOCOL:

All the experiments were performed under the Guidance Suggestions for the Care and Use of Laboratory Animals, formulated by the Ministry of Science and Technology of the People's Republic of China, as well as the principles of the Basel Declaration. Approval was given by the Animal Care Committee of the Sichuan University West China Hospital (Chengdu, China). **Figure 1** shows the single-blind, self-controlled study design used here.

1. rTMS devices

1.1. Use an 8-shaped magnetic field stimulator coil to perform the rTMS stimulation.

2. Animal

2.1. Keep the male rhesus monkey (*Macaca mulatta*, 5 kg, 5 years old) in an individual home cage with free access to tap water and standard chow. Ensure that environmental conditions are controlled to provide a relative humidity of 60–70%, a temperature of 24 ± 2 °C, and a 12:12 h light: dark cycle[14,15]. Perform all the experiments according to the Guidance for the Care and Use of Laboratory Animals.

3. A serial cisterna magna CSF sampling method

3.1. Have two trained experimenters perform a catheterization method to sample CSF from the cisterna magna (**Figure 2**).

3.2. Positioning

3.2.1. Anesthetize the monkey by an intramuscular injection of 0.1 mL/kg zolazepam–tiletamine (see the **Table of Materials**). To ensure successful anesthetization of the monkey, look for deep and slow breathing, dull or absent cornea reflex, and relaxation of the muscles of the extremities.

3.2.2. Place the monkey on an operating table in the lateral decubitus position. Bend the monkey's neck, hunch the back of the monkey, and bring its knees toward the chest.

3.3. Puncture

3.3.1. For disinfection, prepare the area around the lower back using aseptic technique. Insert a spinal needle between the lumbar vertebrae L4/L5, push it in until there is a "pop" when it enters the lumbar cistern where the ligamentum flavum is housed.

3.3.2. Push the needle again until there is a second "pop" where it enters the dura mater. Withdraw the stylet from the spinal needle and collect drops of CSF.

3.4. Catheter insertion

3.4.1. Under X-ray guidance, insert the epidural catheter through the puncture needle into the subarachnoid space until it is buoyant in the cisterna magna.

3.5. Port implantation

3.5.1. Make a 5 cm incision from the puncture site to the direction of the head and isolate the skin from subcutaneous tissue to place the sampling port. Connect the port to the end of the epidural catheter and implant the port under the skin; then, suture the incision. Disinfect the wound daily to prevent infection.

NOTE: The monkey fully recovers on the day after surgery.

3.6. CSF collection

3.6.1. Use the bars of the cage to restrain the monkey and keep its back bent.

3.6.2. Insert a syringe into the center of the sampling port to extract the CSF from the cisterna magna through the catheter. Discard the first 0.2 mL of CSF (the total volume of the catheter and port is 0.1 mL), and then collect 1 mL of CSF for analysis[16].

4. Monkey chair adaptive training

4.1. Fix the monkey on the monkey chair before the experiment to avoid interrupting the process of rTMS intervention (**Figure 3A,B**).

4.2. Collect CSF for biomarker analysis in the awake state of the monkey to avoid the influence of anesthetic drugs.

4.3. On the third day after the subarachnoid catheterization, 2 weeks before the start of the

experiment, subject the monkey to adaptive training with the monkey chair, twice a day, for 30 min each time.

5. rTMS adaptive training/sham stimulation

5.1. Conduct the rTMS adaptive training/sham stimulation one week after the adaptive training with the monkey chair, one week before the start of the formal experiment to avoid hindering the progress of the experiment because of vibrations and sounds during the stimulation process.

5.2. Use a sham coil (which only produces vibration and sound and does not generate a magnetic field) to stimulate the monkey. Offer food to the monkey after stimulation to help it adapt to the process (**Figure 3C**).

5.3. Conduct rTMS adaptive training on a monkey chair twice a day, for 30 min each time for a total of 2 weeks.

6. Treatment protocol

6.1. Use three different frequencies (1 Hz/20 Hz/40 Hz) of rTMS to stimulate the bilateral-DLPFC (R-L-DLPFC) of the monkey, as described previously[17]. Localize the DLPFC according to the international 10-20 system.

6.1.1. Conduct three different sessions of rTMS with a washout period exceeding 24 h[18,19].

6.1.1.1. For the first period, use the following parameters: a frequency of 1 Hz for rTMS, a pattern of rTMS composed of 20 burst trains, 20 pulses with 10 s inter-train intervals between trains, and an intensity of stimulation of 100% of the average resting motor threshold (RMT), twice a day for three consecutive days[20,21].

6.1.1.2. For the second period, use the following parameters: trains of high frequency (20 Hz) rTMS with 100% RMT for 2 s duration with 28 s inter-train intervals, a total of 2,000 stimuli (40 stimuli/train, 50 trains) each session, twice a day for three consecutive days[22].

6.1.1.2. For the third period, use the following parameters: trains of gamma-frequency (40 Hz) rTMS with 100% RMT delivered in 1 s duration separated by 28 s inter-train intervals. Keep the total number of pulses for each session the same as with 20 Hz rTMS, twice a day for three consecutive days[7,22].

7. CSF biomarkers

7.1. Analyze four CSF biomarkers: $A\beta_{42}$, $A\beta_{42}/A\beta_{40}$, tTau, and pTau.

8. CSF collection and index detection method

8.1. Use a minimally invasive catheterization method to sample the CSF.

8.2. Have one operator bend the monkey's neck to bring its knees toward the chest. Instruct the other operator to insert a syringe into the center of the sampling port, ensuring that CSF is extracted through the catheter.

8.3. Collect CSF at 5 timepoints (4 samples each timepoint at 3 min intervals): pre-rTMS, 0 h/2 h/6 h/24 h post-rTMS[23-25]. Collect a total of 60 samples for 3 frequencies; number and store them in a -80 °C refrigerator for up to 1 month. After the experiment, subject all samples to liquid chip detection according to the manufacturer's instructions (see the **Table of Materials**).

9. Statistical analysis

9.1. Present all data as mean ± standard deviation (SD).

9.2. Perform the Shapiro-Wilk test to test normality in case of a small sample size. Perform two-way repeated-measures ANOVA and Tukey's multiple comparisons test.

NOTE: A value (two-tailed) < 0.05 was considered statistically significant.

REPRESENTATIVE RESULTS:

The results showed that rTMS could affect the Aβ and tau levels in rhesus monkey CSF. CSF biomarker levels changed with time after rTMS stimulation at different frequencies (1 Hz, 20 Hz, and 40 Hz).

$A\beta_{42}$ and $A\beta_{42}/A\beta_{40}$

As shown in **Figure 4A**, after 1 Hz rTMS stimulation, the $A\beta_{42}$ levels gradually increased over 24 h ($p < 0.05$) and returned to baseline after the washout period. Similarly, after stimulating the bilateral DLPFC of the monkey with rTMS at 20 Hz, the $A\beta_{42}$ levels increased with time and reached a peak at 6 h after stimulation ($p < 0.05$). However, after stimulation with 40 Hz rTMS, the $A\beta_{42}$ levels significantly increased immediately at the timepoint of post-rTMS ($p < 0.05$) and decreased slowly. In general, the high frequencies of rTMS (20 Hz and 40 Hz) increased $A\beta_{42}$ levels to a greater extent than the low frequency (1 Hz) ($p < 0.05$). Moreover, the $A\beta_{42}$ levels increased more quickly at the high frequencies, especially at 40 Hz, reached a peak just after stimulation. Moreover, the $A\beta_{42}$ level at 40 Hz rose significantly compared with that at 20 Hz ($p < 0.05$). The ratio of $A\beta_{42}/A\beta_{40}$ showed an upward trend after stimulation with 1 Hz and 20 Hz rTMS and significantly increased from 2 h after rTMS stimulation. Further, it increased to a greater extent after 20 Hz rTMS than with 1 Hz ($p < 0.05$) (**Figure 4B**). However, there was no significant difference in the $A\beta_{42}/A\beta_{40}$ ratio at 40 Hz.

pTau and tTau

Overall, the tTau levels in monkey CSF immediately increased after both 20 Hz and 40 Hz rTMS stimulation ($p < 0.05$) and decreased gradually (**Figure 4C**). However, there was no significant

difference after 1 Hz rTMS. The pTau level increased immediately and dramatically after the stimulation with 40 Hz rTMS ($p < 0.05$) and decreased to below baseline level after 24 h (**Figure 4D**). Additionally, the pTau level showed a downward trend after 1 Hz and 20 Hz rTMS stimulation. Therefore, compared to the other two frequencies (1 Hz and 20 Hz), 40 Hz rTMS showed more significant effects on Tau levels ($p < 0.05$).

Baseline after washout

After a 24 h washout period, no significant difference from baseline ($p > 0.05$) was observed in any CSF biomarker levels.

FIGURE AND TABLE LEGENDS:

Figure 1: The flow chart for this pilot study. Abbreviation: rTMS = repetitive transcranial magnetic stimulation.

Figure 2: Minimally invasive catheterization for serial sampling of CSF from cisterna magna. A routine lumbar puncture was followed by a minimally invasive catheterization, in which an epidural catheter penetrated the subarachnoid space and was kept floating in the cisterna magna under the guidance of X-ray (red arrow). A sampling port was left subcutaneously beside the puncture point to allow sampling of the cisterna magna CSF under in a fully conscious animal. Abbreviation: CSF = cerebrospinal fluid.

Figure 3: Monkey chair adaptability training. (**A**) Front; (**B**) lateral; (**C**) rTMS adaptive training/sham stimulation. Abbreviation: rTMS = repetitive transcranial magnetic stimulation.

Figure 4: Effects of rTMS on Aβ and tau levels in rhesus monkey CSF. The five bars for each frequency represent five timepoints: pre-rTMS, 0 h post-rTMS, 2 h post-rTMS, 6 h post-rTMS, and 24 h post-rTMS. (**A**) Changes in $A\beta_{42}$ level in monkey CSF after rTMS; (**B**) changes in $A\beta_{42}/A\beta_{40}$ ratio in monkey CSF after rTMS; (**C**) changes in tTau levels in monkey CSF after rTMS stimulation; (**D**) Changes in pTau levels in monkey CSF after rTMS. * represents a significant difference from the pre-rTMS level, $p < 0.05$. # and ▲ represent significant differences from the level of 1 Hz or 20 Hz at the same timepoint, respectively. $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** represents $p < 0.0001$. Abbreviations: rTMS = repetitive transcranial magnetic stimulation; CSF = cerebrospinal fluid; tTau = total Tau; pTau = phosphorylated Tau.

DISCUSSION:

$A\beta_{1\text{-}42}$, a well-established biomarker of AD, is a CSF core biomarker related to Aβ metabolism and amyloid plaque formation in the brain and has been widely used in clinical trials and the clinic[26]. Recent studies have shown that the CSF $A\beta_{42}/A\beta_{40}$ ratio is a better diagnostic biomarker of AD than $A\beta_{42}$ alone because it is a better indicator of the AD-type pathology[27,28]. Tau and pTau proteins are released into the extracellular space during the neurodegenerative process, resulting in increased tau concentrations in CSF[20,29]. Therefore, CSF $A\beta_{1\text{-}42}$, $A\beta_{42}/A\beta_{40}$, tTau, and pTau are confirmed and combined CSF biomarkers in the revised diagnostic criteria of AD[1,29].

This study demonstrates that after the rTMS stimulation, the $A\beta_{42}$ levels in CSF showed an

upward trend at all frequencies. High-frequency rTMS (20 Hz and 40 Hz) increased the $A\beta_{42}$ levels to a greater extent than the low frequency. According to previous research[30,31], a low level of $A\beta_{42}$ in CSF is associated with AD-specific neurodegeneration (i.e., hippocampal atrophy). However, the increase in Aβ after rTMS stimulation reverses the pathological features of AD, indicating that rTMS may normalize Aβ levels. A preclinical study indicates that the Aβ level is regulated by neuronal activity[32]. Therefore, high-frequency rTMS, vs. low-frequency rTMS, may increase the production of all Aβ substances, including $A\beta_{42}$, by activating neural network activity. In addition, the study found that after 24 h of rTMS at three different frequencies (1 Hz, 20 Hz, and 40 Hz), the pTau level was below the baseline. This indicated a decrease in the abnormal pTau protein, reducing its binding to microtubules and maintaining the normal structure of neurons. However, after high-frequency rTMS, the tTau level of CSF immediately increased and gradually decreased over 24 h. The mechanism underlying this phenomenon is still unclear.

This study objectively confirms the effect of rTMS on Aβ and tau metabolism in CSF. Compared with other evaluation methods, CSF biomarkers can reflect the metabolism and pathology of the brain, providing a window for the brain. This method is safe and well-tolerated and has great clinical applicability[33,34]. The most common technique to collect CSF is to perform a lumbar puncture. However, it is challenging to collect CSF several times in a short period, as there are risks of CNS infection and CSF leakage due to the repeated dural puncture[35,36].

This protocol uses a novel CSF sampling method, allowing for repeated CSF sampling under fully awake conditions, with low risks of the aforementioned adverse events. The sampling port is placed under the skin so that the monkey cannot scratch the port. Therefore, the CSF can be directly collected through the sampling port rather than by lumbar puncture. The method is convenient and quick and avoids the impact of anesthetics[16]. Therefore, researchers who need multiple samples of monkey CSF can consider this serial cisterna magna CSF sampling method.
To avoid interrupting the process of rTMS, monkey chair adaptive training and rTMS adaptive training are important before beginning the experiment.

Nevertheless, the monkey's head still has a small range of movement during the experiment even after the training. Hence, it is advisable to use a robot-assisted tracking system, to localize the stimulation sites and position the TMS coil simultaneously when the head moves. This study has some limitations: the animal used here was a normal monkey rather than a pathological model (such as aged canines[37]), and the sample size was small. However, this pilot study has shown interesting dynamic changes in the levels of Aβ and tau after rTMS, indicating the potential benefits of rTMS on AD and warranting further investigation.

ACKNOWLEDGMENTS:
The authors would like to thank Sichuan Green-House Biotech Co., Ltd for providing the monkey chair and other relative devices. This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

DISCLOSURES:
The authors have no conflicts of interest to declare.

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Figure 1

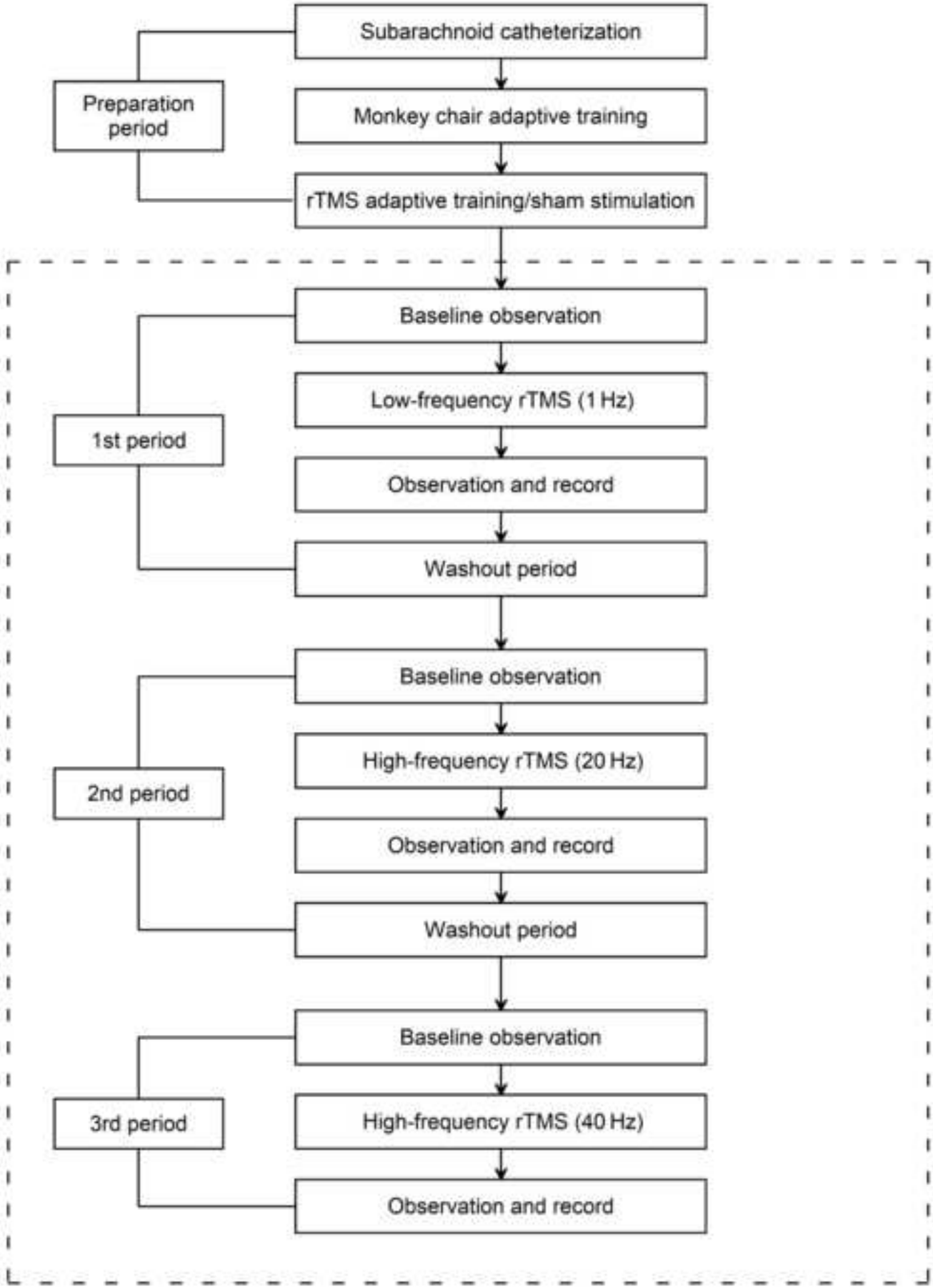

Figure 2

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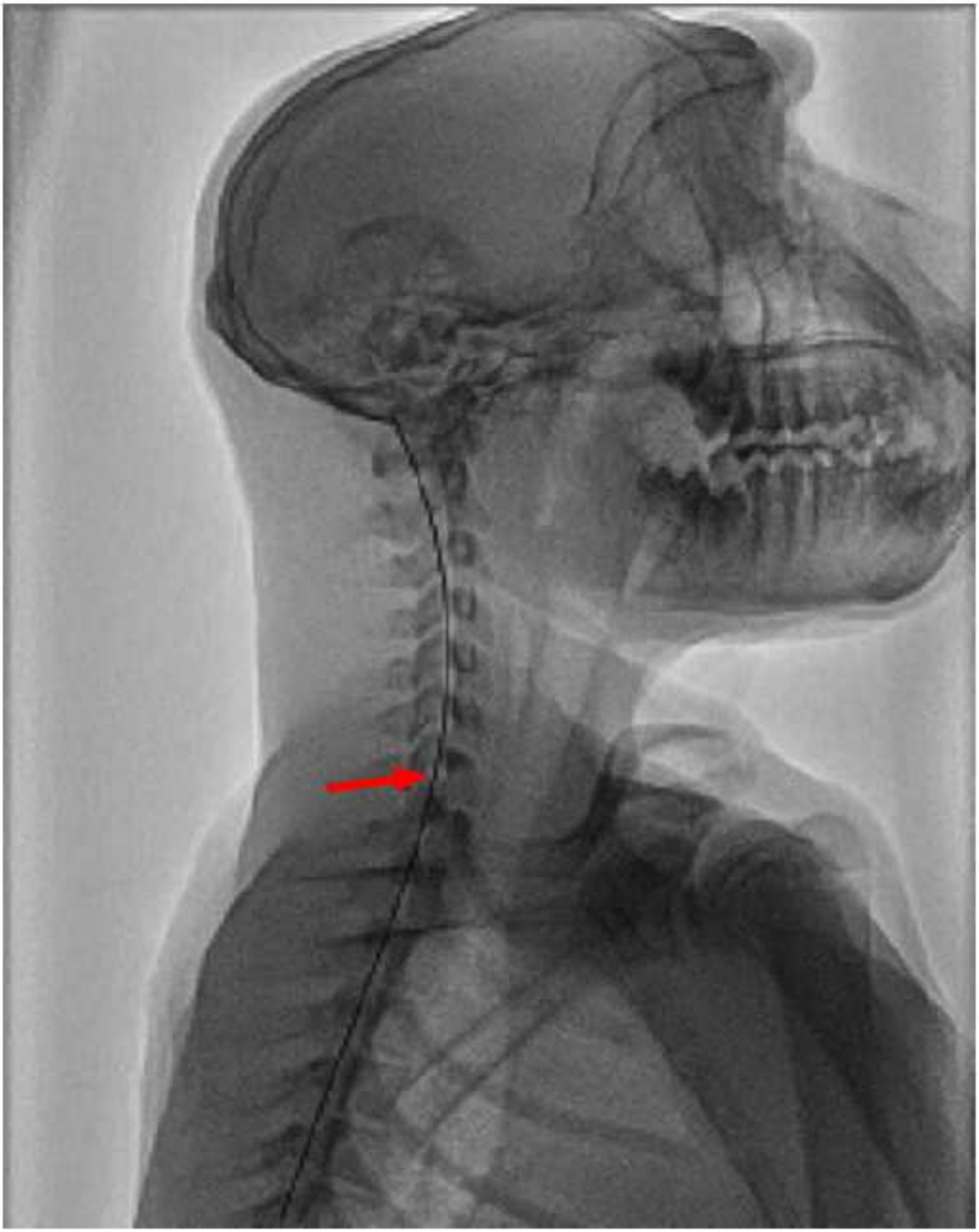
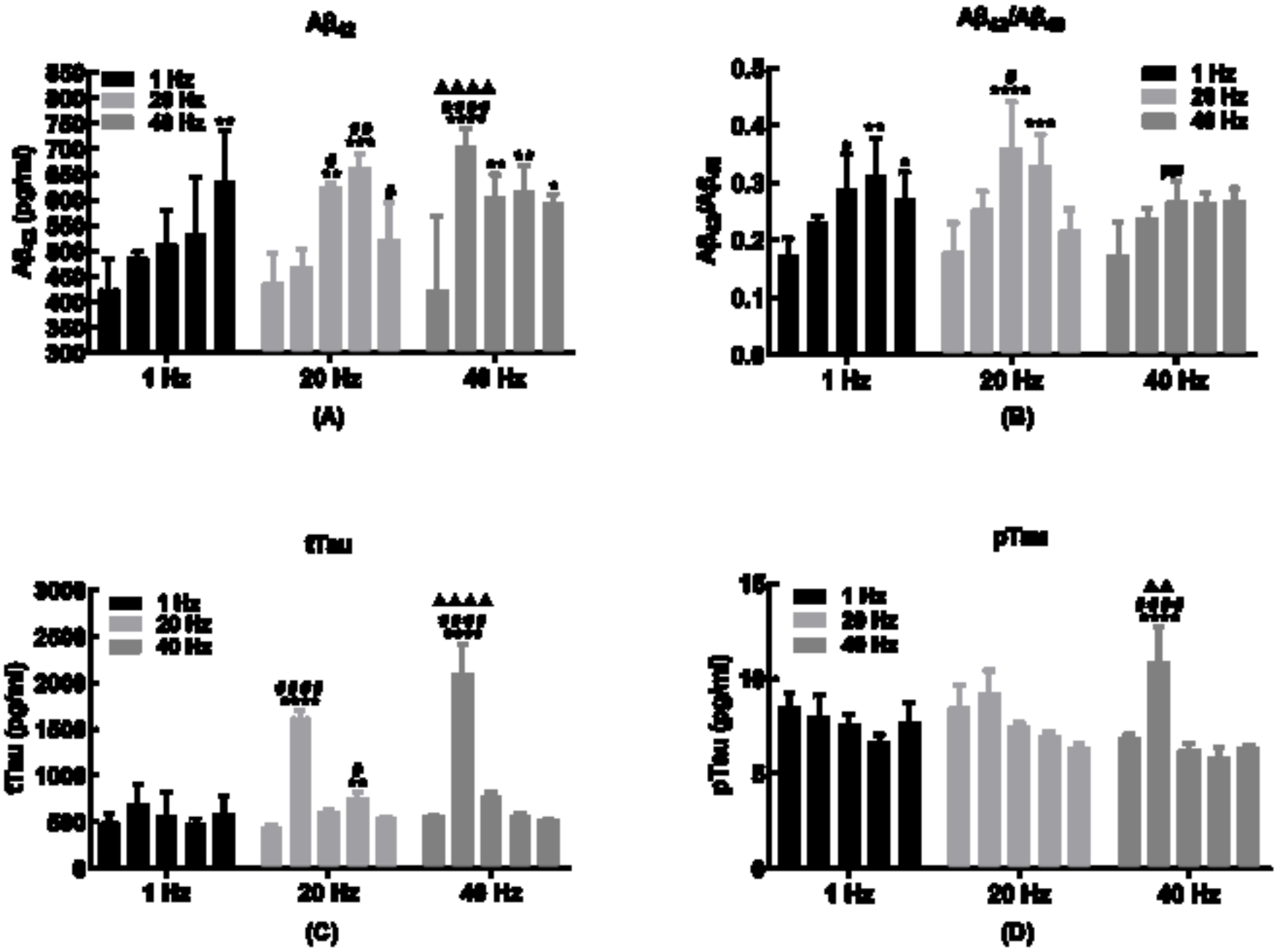


Figure 3

Click here to access/download;Figure;Fig 3.jpg

Figure 4



Click here to access/download

Table of Materials

Materials Table (1).xlsx

-Response to the reviewers' comments

Dear Dr. Nilanjana Saha and Reviewers:

Thank you for your letter and for the reviewers' comments concerning our manuscript entitled "Effects of repetitive transcranial magnetic stimulation on Aβ and tau levels in rhesus monkey cerebrospinal fluid: A pilot study" (Manuscript ID: JoVE63005). Those comments are all constructive and very helpful for revising and improving our paper, as well as the important guiding significance to our researches. We have studied all comments carefully and have made correction which we hope meet with approval. Amendments are marked in red in the revised manuscript. The main corrections in the paper and the responds to the reviewer's comments are as follows:

Reviewer number	Original comments of the reviewers	Reply by the author(s) point by point	Line number of changes
Editorial comments	Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.	We have made every effort to improve the quality and clarity of the language throughout the manuscript. Changes that have been made to the manuscript are denoted in highlight.	
Editorial comments	Please revise the following lines to avoid previously published work: 91-93, 124-132, 133-137.	Thanks so much for your careful check. We have revised these parts to avoid previously published work.	Line 89-91 Line 120-135
Editorial comments	Please try to give a more concise title. It can be something like, "A pilot study on the repetitive transcranial magnetic stimulation of Aβ and tau levels in rhesus monkey cerebrospinal fluid."	We gratefully appreciate for the valuable comment. We have re-written the title according to your suggestion.	Line 2-3
Editorial comments	Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog	Thanks a lot for your kind suggestion. We have revised the table of the essential supplies, reagents, and equipment and sorted the Materials Table	the Materials Table Line 103

	number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material.	alphabetically by the name of the material.	Line 115 Line 189 Line 194
Editorial comments	Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).	Thanks for the rigorous advice. We have revised this manuscript to avoid the use of any personal pronouns.	Line 118 Line 151 Line 186 Ling 260 Line 266 Line 267 Line 285
Editorial comments	JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials (including reagents, instruments, software, etc.). Please sort the Materials Table alphabetically by the name of the material.	Thanks a lot for your kind suggestion. We have revised the table of the essential supplies, reagents, and equipment and sorted the Materials Table alphabetically by the name of the material.	the Materials Table Line 103 Line 115 Line 189 Line 194
Editorial comments	The Protocol should be made up almost entirely of discrete steps without	The Protocol has been revised according to editorial comments.	Line 103-195

	large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.		
Editorial comments	Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly.	The Protocol has been revised according to editorial comments.	Line 103-195
Editorial comments	Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be	We gratefully appreciate for your valuable suggestion. We have added the details so that viewers can easily replicate the protocol	Line 103-195

	enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.		
Editorial comments	Please add more details to your protocol steps: Step 2.1: Please mention the number of trained personnel needed simultaneously to carry out the work. Line 120: Please mention how the successful anaesthetization was ensured. Line 129: how much should the catheter be inserted? Line 137: How the correct protein level was ensured. Line 171: Please include the details of all the biomarkers used in the Table of Materials. Line 186: How long can the samples be stored? Line 187-188: Please provide generic terms for the detection kit and software. Details should be included in the Table of Materials.	Thanks so much for your careful check. Line 113: there are 2 trained experimenters needed to carry out the work. Line 115-117: To ensure the successful anaesthetization, the state of the monkey should be breathed deeply and slowly, cornea reflex is dull or disappeared and muscle relaxation of extremities. Line 125-126: Under the guidance of X-ray, the catheter should be inserted until it could be buoyant in cisterna magna. Line 134-135: In this study, we discard the first 0.2ml CSF (the total volume of the catheter and port is 0.1ml), and then collect 1ml CSF for analysis. Line 171: All the biomarkers were tested by Human Amyloid Beta and Tau Magnetic Bead Pane l which have been included in the Materials Table. Line 188: The samples can be stored no more than 1	Line 113 Line 115-117 Line 125-126 Line 134-135 The Materials Table Line 188 Line 189

		month. Line 189: We have included the details of the detection kit and software in the Materials Table.	
Editorial comments	Please include a one-line space between each protocol step and then highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Also, please ensure that it is in line with the title of the manuscript. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.	Thanks for your rigorous comment. We have included a one-line space between each protocol step and then highlighted up the Protocol in yellow that identifies the essential steps of the protocol for the video.	Line 112-189
Editorial comments	Please modify the Result section to include all the observations and conclusions you can derive from the Figures.	Thanks for your nice suggestion. We have added the observations from the figures in the *REPRESENTATIVE RESULTS*.	Line 208-211 Line 213-215
Editorial comments	As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations: a) Critical steps within the protocol b) Any modifications and	We gratefully appreciate for your valuable suggestion. We have revised the *Discussion* according to your advice.	Line 266-289

	troubleshooting of the technique c) Any limitations of the technique d) The significance with respect to existing methods e) Any future applications of the technique		
Editorial comments	Figure 4: Please provide a space between the number and the unit, such as “1 Hz”, “20 Hz”, etc. Also, in the description of the y-axis, please include a gap between the number and the unit.	Thanks so much for your careful check. We have revised the Figure 4 according to your comment.	Figure 4
Editorial comments	Please spell out the journal titles in the References.	Thanks so much for your careful check. We have spelt out the journal titles in the References.	Line 304-397
Reviewer #1	Method to fix the catheter need to be described further with a picture of the site.	We gratefully appreciate for the Reviewer’s valuable comment. However, we are very sorry that we did not take a photo of the site. We added some details about this step to help the reader understand it.	Line 127-130
Reviewer #1	Training to adapt rTMS seems good. However, localization of DLPFC should be described more precisely. And if the response of the monkey could be explained, it will be better.	Thanks for the Reviewer’s rigorous advice. The localization of DLPFC has been described in Line 156-157.	Line 156-157

Reviewer #1	In Fig. 4, an increase in tTau and pTau is suggested. How about present the ratio of pTau/tTau? The 20Hz stimulation of tTau seems to increase in the post rTMS stimulation, however, since there is no change in the amount of pTau, this may mean a decrease in pTau. In addition, both tTau and pTau are increased by post-rTMS stimulation at 40Hz stimulation. If the ratio is calculated, this increase can be insignificant.	Thanks for the Reviewer's rigorous comment. Niemantsverdriet *et al* reported that the three CSF biomarkers $A\beta_{1-42}$, T-tau, and P-tau_{181} are strongly associated with future development of AD dementia. Janelidze *et al* found that the CSF $A\beta_{42}/A\beta_{40}$ ratio was significantly better predictors of abnormal amyloid PET than CSF $A\beta_{42}$. However, Grossman *et al* demonstrated that the CSF level of the ratio of pTau/tTau may be a candidate biomarker to provide objective support for the diagnosis of ALS not AD.	None
Reviewer #1	It seems necessary to revise the notation of the contents of Fig. 4 for pre-rTMS, post-rTMS, 2h post rTMS, 6h post-rTMS and 24 post rTMS. If the meaning of Post-rTMS is immediately after processing, it seems that 0h should be added (0h post rTMS).	Thanks so much for the Reviewer's careful check. We have added 0h (0h post rTMS).	Line 187 Line 242
Reviewer #1	A detailed description of the lipid chip assay is required, and it is likely that details such as product catalog numbers should be entered.	Thanks for the Reviewer's nice suggestion. We have added this part in the Materials Table.	The Materials Table
Reviewer #2	Do the study randomize the stimulation order for 1, 20, and 40Hz in one monkey? How make sure that the rTMS did produce interference effect or after effect	We gratefully appreciate for the Reviewer's valuable comments. In this study, we did not randomize the stimulation order for the three frequencies as we described in Figure 1. We	None

	among each frequency intervention? How to avoid the accumulation effects of rTMS when testing different rTMS parameters in one animal?	collected CSF at 5 time points (pre-rTMS, 0H/2H/6H/24H post-rTMS) to explore interference effect or after effect of rTMS stimulation at different frequencies. A washout period exceeding 24h was used to avoid the accumulation effects of rTMS.	
Reviewer #2	The head of money should move freely during rTMS intervention since no firmly fixation of head. How to mark sure the stimuli of rTMS can precisely target to the DLPFC during the long period of brain stimulation?	Thanks for the Reviewer's rigorous comment. We did rTMS adaptive training before the formal experiment so that the monkey did not be scared because of the vibrations and sounds during the stimulation process. Nevertheless, the monkey's head still has a small range of movement during the experiment even after the training. It would be better if the robot-assisted tracking system can be used, which can localize the stimulation sites and position the TMS coil simultaneously when the head moved.	Line 281-284
Reviewer #2	How to localize the DLPFC of Money? According the international 10-20 system?	Thanks for the Reviewer's rigorous advice. We have added the details according to the Reviewer #1.	Line 156-157
Reviewer #2	Why the study used the statistical approach of one-way ANOVA?	As we described in Statistical analysis, we used two-way repeated-measures ANOVA not one-way ANOVA.	None

We have studied reviewer's comments carefully and have made revision which marked in red in the paper. We have tried our best to revise our manuscript

according to the comments. Attached please find the revised version, which we would like to submit for your kind consideration.

We would like to express our great appreciation to you and reviewers for comments on our paper. Looking forward to hearing from you.

Thank you and best regards.

Yours sincerely,

Ling-Yi Liao

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